Use of Biological Agents to Enhance the Preservative Treatment of Electrical Distribution poles

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners

Signed..... [redacted] ..... Date 30/10/98

Director of studies

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ABSTRACT

Treatment of timber with preservative varies from species to species. Poor preservative penetration has been associated with wood decay and shortened service life of treated timbers. The preservation of Sitka spruce and Scots pine heartwood by conventional methods is poor with the depth of preservative penetration being only a few millimetres.

The use of *Trichoderma* isolates to improve the permeability of such timbers provides a novel approach to timber treatment. Initial studies established that *Trichoderma* isolates varied in their ability to produce selected extra-cellular enzymes (cellulase, pectinase and amylase) which would be important in the improvement of the permeability of Scots pine and Sitka spruce material.

On the basis of the ability of the organisms to produce extra-cellular enzymes *in vitro*, 5 isolates were selected and tested against dried and freshly cut small wood block samples. The high moisture content of green wood is recognised as being inhibitory to some colonising organisms. Results indicated that selected isolates were capable of growing through fresh wood samples and varied in their ability to improve the permeability of the samples.

Methods best suited to determine the changes in permeability were developed and assessed. Two methods were finally used: a fluid uptake and an air flow method. Both methods were used to demonstrate the ability of the isolates to improve the permeability of Scots pine and Sitka spruce material.

Two *Trichoderma* isolates showed a consistent ability to improve the permeability of pine and spruce material. These isolates were used to inoculate freshly felled

roundwood log sections. The two *Trichoderma* isolates successfully colonised and improved the permeability of the timber.

When taken into pole material the isolates continued to show improvements in the permeability and subsequent preservative penetration. Analysis of the preservative treatment of *Trichoderma* treated logs showed deeper penetration and higher loadings than in untreated control logs. In spruce the loadings in the preservative treated zone were increased by approximately 300% although this was still below the minimum retentions required by the electricity supply industry. Pine material showed increased loadings in the treated zone with the highest loadings being found in the outer regions of the pole material.

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Chapter 1. Introduction

1.1 Background

The United Kingdom imports almost 90% of its timber requirements with lumber being the UK's second largest import (Price, 1989). The annual requirement for pole material (Electricity Supply Industry and British Telecom) in the UK is around 130 000 poles of which around 50 000 are supplied from native pine forests (Aaron and Oakley, 1985). In 1988 the total cost of poles was around £2, 000, 000 to the end users (Harding, 1988). It is obviously desirable for end users to reduce this expenditure by either extending the service life of the poles or by using alternative cheaper wood species.

Scots pine (*Pinus sylvestris*) is the most commonly used timber species for the production of distribution poles in the UK and has become the yardstick for assessing the performance of other species. Scots pine has good strength properties and its dry sapwood readily absorbs preservatives giving a more than adequate penetration and retention (Aaron and Oakley, 1985). However the yield of poles per hectare in the forest is generally low because of poor form (straightness). When pole failure occurs with Scots pine it is generally due to decay in the impermeable heartwood region which is untreated with preservative.

Since the establishment of the Forestry Commission in 1918 some 90% of UK planting has been Sitka spruce (*Picea sitchensis* (Carr) Bong). This fast growing timber species was introduced from the North West coast of the United States in 1831. This species, removed from its natural pests has thrived under the UK climate and produces a moderately good yield of poles per hectare. However this more readily available timber species may not always display the appropriate characteristics required for distribution poles. Sitka spruce has a relatively lower strength than Scots pine. When used for transmission poles, spruce material is generally stouter than the same length of Scots pine poles. Sitka spruce has not been used

extensively by the electricity supply industry because the resulting poles are difficult to treat by using the usual pressure impregnation methods (Harding, 1988). Under normal treatment processes the observed penetration of preservative is only a few millimetres into the sapwood (a depth of 50mm is generally required). Scots pine treatment normally gives complete sapwood penetration as required by the Electricity Supply Industry (BS1990).

With the poorer penetration of preservative into Sitka spruce material, only the outer layers of the wood will be protected. If the timber remained intact adequate protection could be gained from the preservative treatment. However on drying spruce timber will split (check) and wood beyond the preservative treated layer can be exposed leaving the timber at risk of decay in this region (Hainey, 1992).

1.2 Wood Structure

Wood is a complex material that is composed of a series of inter-connected elements that run in longitudinal and radial directions. These elements take different forms and structures. Within the living tree these elements are utilised for different functions. Longitudinal elements in softwoods (tracheids) are a series of cells that are connected by a number of bordered pits that regulate the flow of fluids through cells (Browning, 1975). The size, shape and number of pits per tracheid varies from species to species and is dependant on when the cells were laid down. Early wood (material laid down during periods of rapid tree growth e.g. spring and summer) generally contains a larger number of pits than late wood (material laid down during other seasons). Typically early wood contains between 50 and 300 pits per tracheid (Siau, 1984). The function of these tracheids is to transport fluids and chemicals longitudinally throughout the tree and to add strength and structure to the tree. Longitudinal tracheids constitute about 90% of the wood volume and are therefore largely responsible for the resulting physical and chemical properties of softwoods. In most softwoods the length of an individual tracheid ranges from 3-5mm, but this varies from species to species, e.g.

redwood (*Pinus sylvestris*) can have tracheids up to 7.3mm long and cedar up to 1.7mm (Thomas, 1977).

Medullary rays and resin canals penetrate into the wood in a radial direction. These rays are linked to longitudinal tracheids and can transport materials in solution radially to and from the cambium and inner bark. The rays also serve as storage sites for carbohydrates, particularly starch which is only partially soluble (Browning, 1975).

Within mature wood there are normally two distinct zones, these being sapwood and heartwood. Histologically, heartwood and sapwood are almost identical, the only changes that take place during the transformation from sapwood to heartwood are the aspiration of bordered pits and deposition of extraneous material that encrusts the closed pits (Krahmer and Cote, 1963; Jane, 1970).

Sapwood is composed of living cells, which continue to be laid down throughout the life of the tree. This zone is where sap is conducted and carbohydrate reserves are stored and provides support for the crown of the tree (Browning, 1975). Heartwood is generally impermeable to liquids because pit closure during drying and the deposition of extraneous material in the cells block bordered pit membranes preventing the movements of fluids and other materials. Heartwood is physiologically dead and mainly offers support to the tree. In some species the heartwood is thought to be fairly durable because the high concentrations of extractives deposited in this region can prevent or partially inhibit the growth of decay organisms (Rudman, 1962; Rudman, 1963)

1.3 Wood permeability

During seasoning timber dries down to a moisture range appropriate to the conditions and purposes for which it is to be used (ESI, 43-88). As timber dries, changes occur in the wood that affect its permeability. Pit membranes aspirate (close) which dramatically reduces the permeability of the timber and subsequent preservative penetration (Phillips, 1933; Banks, 1970).

1.3.1 Sapwood Permeability

Sapwood by its nature is generally more permeable than heartwood, and there is a vast difference in the permeability of different species due to their anatomical differences. The permeability of the wood is influenced by: the numbers and types of bordered pit membranes found in the tracheids; the physical state of the membrane and the length and diameters of the tracheids and ray cells within the wood (Elliot, 1960; Liese and Bauch 1967; Banks, 1970; Petty, 1970; Siau, 1984). Refractory species like Douglas fir (*Pseudotsuga menzii*) and Sitka spruce are known to be more difficult to treat with preservative than other species (Liese and Bauch, 1967; Liese, 1967; Bauch *et al*, 1970) .Sitka spruce has a lower permeability because of the structure, number and condition of pit membranes in the timber. With spruce material bordered pits are generally smaller than pine material, the shapes of the pits are such that spruce material is more likely to aspirate compared with pine material (figure 1.1).

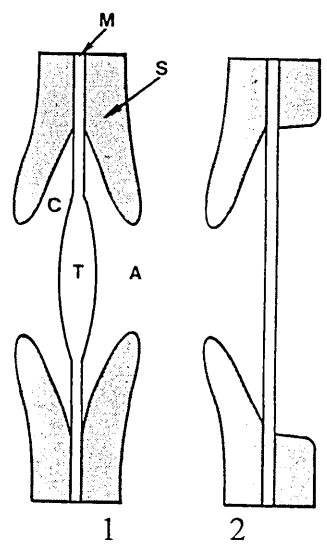


Figure 1.1 Shape of bordered pits in spruce (1) and pine (2) material. N.B. "M" represents the primary call wall. "S" represents the secondary cell wall. "C" represents the chamber. "T" represents the pit torus. "A" represents the pit aperture.

The physical state of the pit membranes has a large impact on the permeability of the wood. Pit membranes are composed of a torus that is connected to the pit edge by cellulose based microfibrils. When open the torus is held in the middle of the pit allowing sap to flow around the edge of the membrane. If the tree is damaged (wounded) and air enters the system, pits close (aspirate) as the pressure behind the pit drops and the pit is pulled tightly shut against the pit border (Siau, 1984). The structure of a pit membrane and the action of aspiration can be seen in figure 1.2.

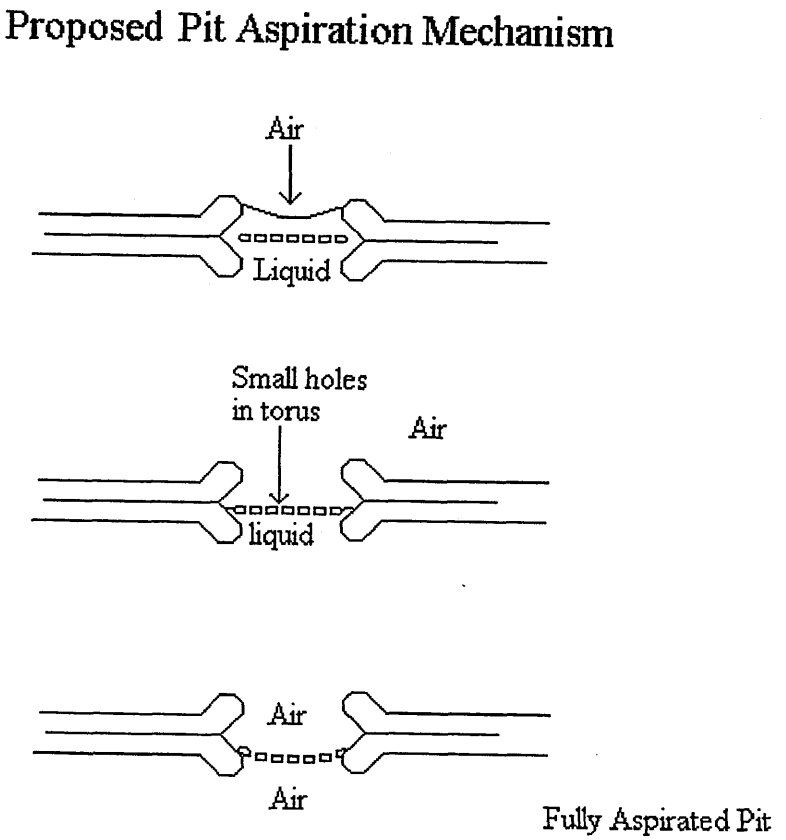


Figure 1.2 Pit membrane and aspiration

When timber is seasoned (air or kiln dried) the permeability of the timber can be reduced to between 1 and 5% of that for green wood depending on the intensity of the heat used to dry the timber (Siau 1984). Aspiration is the cause of most of the problems experienced with the treatability of timbers with preservative.

Scots pine sapwood is more permeable than spruce and hence is more readily treatable, however the heartwood of pine has a lower permeability because of the closure (aspiration) and encrustation of pit membranes in this region (Hillis,1987), resulting in little or no preservative treatment of heartwood.

1.4 Preservation

Traditional methods of preserving timber poles with creosote involves impregnation of preservative under high pressure using an empty cell (Reuping) process. Timber is placed into a treatment chamber which is then pressurised before the preservative is added. The pressure inside the chamber is then increased to force the preservative into the timber, a vacuum is then applied and the excess preservative drawn out of the timber (Nicholas and Siau, 1973). This process can only be carried out on seasoned timber and timbers with a high natural permeability e.g. Corsican pine (*Pinus nigra* var. maritima). Traditional preservation methods are not suitable for all timber species because of the different structures of these timbers.

Preservative treatment is limited by these structural differences and it is the number, size, shape and state of tracheids and pit openings in the timber that are the main influences on how successfully the timber will be treated. There is a strong correlation therefore between the treatability of timbers and the permeability of the wood, i.e. more permeable timber will be treated more easily producing a product which will give a much longer service life.

1.5 Permeability Enhancement

The enhancement of sapwood permeability of refractory wood species by artificial methods has continued to interest researchers through the years and many articles have reported varying levels of success in improving preservative treatment in processed wood (Bauch *et al*, 1970).

There has been a large amount of work undertaken on different methods to enhance permeability by either preventing pits closing on drying or deaspirating the pits once drying is complete. Historically six different approaches have been examined.

1.5.1 Solvent Exchange Drying

Early work focused on chemical methods where sap was replaced with a solvent (solvent exchange drying). Various workers (Griffin, 1919; Erikson and Crawford, 1959) showed that when tree sap was exchanged with alcohol and subsequently dried aspiration of the bordered pit membranes was prevented. This relies on the solvents being miscible with water and not promoting hydrogen bonding between the pit membrane and pit border during subsequent drying, thus leaving the torus in the open position and consequently the permeability remains high (Petty, 1978). The use of solvents to de-aspirate pits has had limited success and the longer the material has remained in a dried form the less likely it is that the aspiration can be reversed by solvent exchange drying (Johnson, 1967).

1.5.2 Steam Treatment

It has been suggested that during seasoning of steamed wood less aspiration occurs. Nicholas and Thomas (1968(a)) showed that steam treatment of Loblolly pine damaged the pit membranes, preventing aspiration. Steaming timber in a green state has been shown to give the best results as the permeability of this wood after seasoning remains higher than in unsteamed material. The drying rate of steamed timber has also been shown to increase, as the more permeable timber loses water faster. However this process has the disadvantage that at high temperatures the structure of the wood can be disrupted and the impact strength reduced with soluble structural components being leached from the timber (Dunleavy and Fogarty, 1971),

1.5.3.Chemical treatments

By using chemical treatments it is conceivable that wood could be treated to increase pore size or reduce aspiration in the seasoning timber. Some researchers report increases in liquid penetration after treatment with sodium chlorite, acids and bases (Nicholas and Siau, 1973). However these treatments resulted in excessive reductions in the strength of the timber. Other chemical treatments have involved extracting some of the extraneous material that is deposited on the bordered pit membranes in the sapwood and heartwood (Nicholas, 1976). Extracting wood with hot water and organic solvents has been shown to improve the permeability of timber samples and has been linked to the removal of material deposited in resin canals and other structures but not from the encrusted pit membranes (Krahmer and Cote, 1963). Extraction with hot water may also have a similar effect to steaming the timber. Although the removal of the soluble material may improve the permeability of the timber it is unlikely to be commercially viable, given the amounts of material to be processed and the time required in which to improve the permeability of the timber.

1.5.4 Physical methods

Incising has also been used to improve the penetration of the preservative. This wood is ruptured by mechanical blades to permit the flow of liquids deeper into the wood. This method has the advantage of being cheap but penetration beyond the depth of the incision remains the same, and the method also results in the loss of some strength (Banks, 1973).

1.5.5 Enzymic degradation

Given that certain enzymes may be capable of breaking down some of the structures responsible for aspiration within the wood, a pretreatment of the timber with a solution of these enzymes may prevent aspiration occurring or de-aspirate the pits after closure. The use of cellulase (Madan and Mohindra, 1981; Ghosh and Ghosh, 1992)and pectinase (Nicholas and Thomas, 1968 b; King and Eggins, 1973; Sharma and Kumar, 1979; Siau,1984) has been documented by several workers. Cellulase was considered by these authors (Madan and Mohindra, 1981; Ghosh and Ghosh, 1992) since the microfibrils supporting the torus are composed of a crystalline cellulose and degradation of these structures would prevent the membrane from closing as the timber seasoned (Jane, 1970). The use of pectinase also improves the permeability of the timber as the torus in the bordered pit membrane is composed mainly of pectin (Nicholas and Thomas, 1968 (b); Bauch *et al*, 1968). This means that even if the pit membrane is aspirated it can be degraded by the enzymes and hence re-opened. The major drawbacks of this approach lie with the application of the enzyme into the timber. It is reasonable to assume that timbers that have a low uptake of preservative will also have a low uptake of the enzyme preparations thereby limiting the effectiveness of the treatment (Imamura *et al*, 1974). Pectinase treatments of heartwood material, even following solvent extraction does not appear to alter the state of the pit membrane. This is attributed to the encrustation of the pit membranes with extraneous material which either prevents the action of the enzyme on the torus by inhibiting it or prevents contact with the torus. (Johnson 1967).

1.5.6 Biological methods

Previous work has shown that the preservative penetration of spruce timber can be improved by ponding the timber prior to seasoning (Dunleavy and McQuire, 1970; Bauch *et al*, 1970; Dunleavy and Fogarty, 1971; Fowlie and Sheard, 1983.). This process relies on the growth of bacteria in the wood and their ability to degrade the structures that restrict permeability and thereby limit preservative penetration into the wood.

The permeability of timber species has been improved after colonisation by a mould fungus (Schultz, 1955). This mould was identified as a *Trichoderma* species, a common soil inhabiting fungus. Other researchers have also examined the use of *Trichoderma* spp. on various timbers including: sapwood of Loblolly pine (*Pinus taeda* L.) (Chidester, 1942); Red Alder (*Alnus rubra* Bong.) (De Freitas and Erickson, 1969); Black Spruce (*Picea nigra* (Ait.) Link) and Aspen (*Populus tremula* L.) (Schulz, 1955); Southern yellow pine (Lindgren and Harvey, 1952); Douglas-Fir (*Pseudotsuga menziesii* var. glauca) (Graham, 1954; Lindgren & Wright, 1954; Johnson & Gjovik 1970).

The use of this fungus has several potential advantages over the ponding method:

i) The use of fungi does not necessitate ponding and hence may be combined with the normal seasoning process for pole material;

ii) The pole material may require a shorter seasoning time compared with ponded materials;

iii) The risk of pre-treatment decay may be reduced by faster seasoning times and possible bio-control effects of the *Trichoderma*.

Biological agents will grow through timber using the available nutrients for growth and breaking down structures within the wood. Depending on which structures are broken down in the wood, organisms can improve the permeability of the material and not adversely affect the nature of the end product. Different organisms will break down wood structures using different enzymes, and the ability to produce these enzymes will vary between different species and isolates of the same organism.

To improve the permeability of pole material the organisms used should produce enzymes that will degrade the structures limiting permeability but not adversely affect the strength of the pole. The enzymes that are likely to be important in this process are: pectinase, cellulase and amylase. Pectinase will degrade the pit torus that is composed mainly of pectin and is held in place by a series of cellulose microfibrils (pit margo). Cellulase may degrade the microfibrils that hold the pit torus in place and prevent aspiration. Amylase will degrade starch which is stored in the ray material and which limits the radial permeability within the timber. (McQuire, 1970)

The heartwood of most timber species is physiologically dead, and is the site of deposition of metabolic bi-products. It is the presence of such chemicals that gives rise to the natural durability of the timber. If *Trichoderma* isolates are to grow through the heartwood of timber to improve the permeability of the material then the isolates must be able to grow in the presence of the chemicals deposited there.

The ability of chemical constituents to confer decay resistance particularly to the heartwood of many timber species is well documented (Jane, 1970; Desch 1981). Scheffer and Cowling (1966) noted that this inherent resistance varied slightly between and within trees of the same species, greater differences were obvious when comparing different wood species. The reason for this inter-species variation in heartwood durability is due to the variety of different chemicals which are deposited during heartwood formation. These include terpenoids, tropolones, flavenoids, stilbenes and other aromatic compounds (Zabel and Morrell, 1992).

Other tree species may contain additional compounds. It has become common practice to categorise both hardwood and softwood timbers on the basis of their natural decay resistance and a current European standard (BS EN 350 pt 2; 1994) classifies wood species on their natural durability and treatability.

While *Trichoderma* isolates have been shown to grow in the heartwood of standing distribution poles (Bruce, 1983) little research has been undertaken to date to examine the inhibitory effect of fresh heartwood extractives on these mould organisms.

Almost all the previous work which has examined the anti-microbial properties of wood extractives has concentrated on heartwood extractives and has examined their inhibitory effects against wood decay basidiomycetes. This work has been used as a means of determining the likely durability of such timbers and also in an attempt to develop new more environmentally acceptable preservatives based on these naturally produced anti-microbial chemicals (Clark *et al*, 1990). Little work has however been reported on the effect of wood extractives against mould colonisers of freshly felled and processed wood products. With increasing recent interest in the development of biological control systems for wood protection and recurrent attention on the use of specific moulds as permeability enhancing agents for refractory timber the effect of chemical extractives on these organisms has assumed greater significance.

1.6. *Trichoderma* .

Trichoderma spp belong to the phylum Ascomycetes and the taxa *fungi imperfecti.* Although Ascomycetes can generally reproduce sexually and asexually, fungi belonging to the taxa

Fungi imperfecti will not reproduce sexually (Alexopoulos *et al,* 1996). The life cycle of an Ascomycete can be seen in figure 1.3, and it should be notes that *Trichoderma* will not produce ascospores and hence will only follow the asexual process (Weisz, 1982).

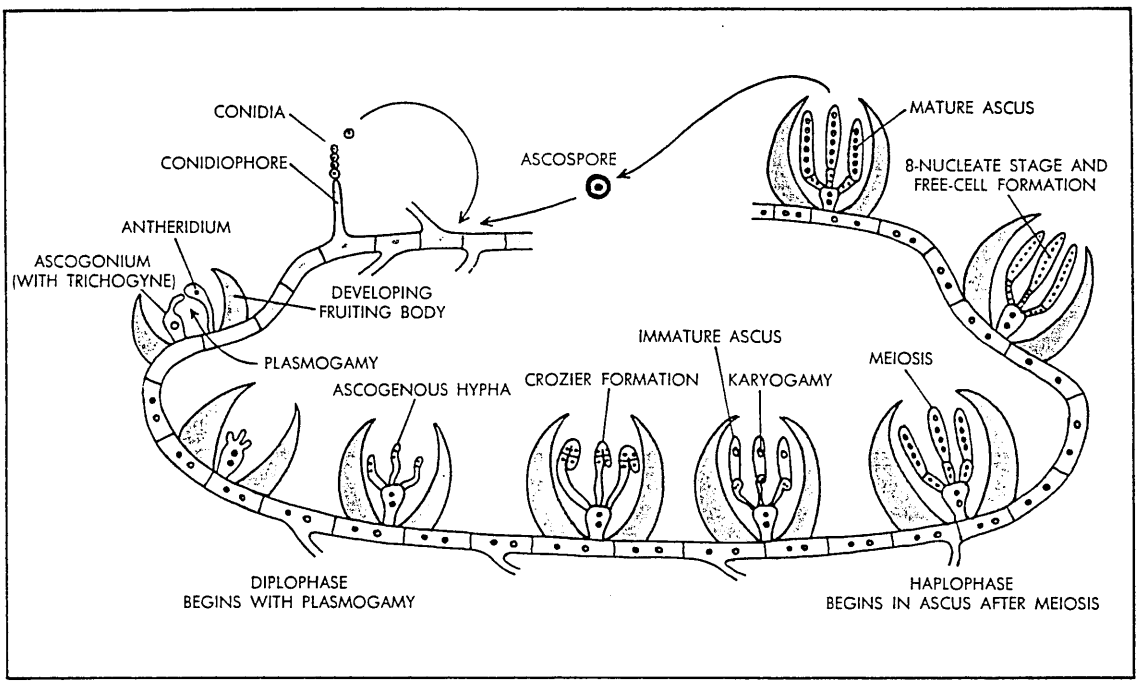


Figure 1.3 The life cycle of *Euascomycetes* (After Weisz, 1982). N.B. Read anti clockwise starting at top centre.

The morphology of the fungus is distinctive and can be recognised by their highly branched conidiophores which bear phialides either singly or in groups.

Trichoderma is a ubiquitous soil fungi that produces white, yellowish or green colonies when cultured. *Trichoderma* spp are generally fast growing pioneer species that are found world wide in most climatic zones (Danielson and Davey 1973; Domsch *et al.,* 1980). They are particularly prevalent in the humid litter of mixed hardwood forests. (Papavizas, 1985)

The widespread occurrence and effective colonisation potential of *Trichoderma* spp. can be associated with several factors including their metabolic versatility; resistance to microbial inhibitors; and their antagonism to other organisms. It has also been noted that *Trichoderma* spp. occupy a pioneer role in nature, this is illustrated in their ability to rapidly colonise newly disturbed sites e.g. fumigated soil, and freshly cut wood stumps. *Trichoderma* spp. can colonise these sites to the exclusion of other fungal species (Schoeman *et al,* 1994).

Recently there has also been a very significant increase in the amount of research into biological control of wood decay fungi as an additional strategy to the use of chemical preservatives for wood protection (Freitag *et al.* 1991; Bruce 1992; Bruce 1997). This has mainly been due to the need for the wood preservation industry to develop more environmentally acceptable technologies for wood protection at a time of heightened public concern on environmental issues. Of those organisms that have been examined as potential biological control agents *Trichoderma* spp. have received greatest attention (Ricard *et al*, 1969; Hulme and Shields, 1970; Smith *et al*, 1981; Bruce & King 1986a, 1986b; Morris et.al., 1986; Bettuci *et.al.*, 1988; Highley & Ricard 1988; Giron & Morrell 1989; Doi & Yamada 1991).

Research has shown that *Trichoderma* isolates are able to control decay by a variety of basidiomycetes in soil block and agar test systems and has highlighted the various control mechanisms which the organisms may employ (Tucker et al,1997). Little has been done however to develop appropriate delivery systems for the control organisms or to establish the

conditions *in-situ* which will determine the extent of *Trichoderma* colonisation and long term survival. Though many authors have reported *Trichoderma* to be a ready coloniser of the sapwood of freshly felled timber including pine species (Butcher, 1968; Dowding 1970) few researchers have examined the ability of *Trichoderma* to colonise heartwood of felled timber. Bruce (1983) established however that *Trichoderma* species can colonise the heartwood regions of Scots pine distribution poles.

Fungi in this genus generally produce extra-cellular enzymes and can antagonise other fungi by a variety of different mechanisms. *Trichoderma* spp., have been seen to colonise timber by using non structural carbohydrates for growth. It is generally accepted that for most *Trichoderma* isolates their ability to degrade timber (ligno-cellulose) is relatively poor. *Trichoderma* spp. have been used commercially for the production of extra-cellular enzymes like cellulase and pectinase (Priest, 1984), and should therefore be capable of disrupting the structures within wood that limit permeability while not adversely affecting the strength of the timber. Although *Trichoderma* has been shown to colonise and improve the permeability of sapwood material in some species, no work has examined its use on Sitka spruce or indeed whether similar colonisation and/or permeability enhancement of fresh heartwood material is possible.

1.7 Aims and Objectives

In order to use *Trichoderma* isolates to improve the permeability of timber, it is necessary to understand the growth patterns and requirements of the organism. Since *Trichoderma* isolates are pioneer organisms they are capable of growing on freshly disturbed sites, and will colonise material quickly, often at the exclusion of other organisms (Bliss, 1951). In order to utilise the nutrients found on disturbed sites the organisms must produce a range of enzymes and be capable of growing in the environment in which they are now placed.

The overall aim of this study was to investigate the effect of selected *Trichoderma* isolates on the permeability of Scots pine and Sitka spruce timber to creosote. This was investigated with the following objectives:

Since *Trichoderma* is such a large genus it was unlikely that all isolates would be capable of growing through timber and improving the permeability of the material. Hence it was necessary to select isolates of *Trichoderma* and test these isolates as permeability enhancers in different timber types.

The first stage of the process was to examine *Trichoderma* isolates for extra cellular enzyme (cellulase, pectinase and amylase) production, as a means of screening strains as potential enhancers of wood permeability.

Once the enzymic capabilities of the *Trichoderma* isolates had been established, the most copious producers were tested in small scale trial to determine the efficiency of selected strains to increase permeability of small wood blocks. In order to assess the efficiency of the screening process a single isolate which does not produce high amounts of the enzymes was also tested for permeability enhancement in wood.

If isolates are to be capable of growing through timber they must be able to grow in the presence of any compound that is found in these timbers. Hence a further objective was to determine the effects of water soluble wood extracts on the growth of the selected *Trichoderma* isolates.

Once selected isolates have been shown to be capable of growing in the presence of wood extracts and improving the permeability of small wood blocks. Suitable methods were developed to measure permeability enhancement in larger samples treated with these isolates. Hence a further aim of the investigation was to develop a suitable method to quantify permeability enhancement in cores removed from treated roundwood logs.

If *Trichoderma* isolates are to be used on a regular basis to improve the permeability of timber it is essential that the effectiveness of different *Trichoderma* delivery systems is investigated.

Once *Trichoderma* isolates were effectively established in selected timbers it was possible to investigate the effect of pretreatment with selected *Trichoderma* isolates on the penetration and retention of creosote into Sitka spruce and Scots pine pole sections. If penetration and retention can be improved then the potential service life of treated poles can be extended, and more extensive end uses for treated Scots pine and Sitka spruce sapwood and heartwood can be envisaged.

Before *Trichoderma* isolates can be accepted as permeability enhancers for the industrial treatment of wood poles it is essential to produce information on the performance of the relative isolates under field conditions in large dressed logs which will be subsequently treated using standard commercial creosote treatment systems. The final objective of the project therefore was to assess the selected strains on large dressed logs of Scots pine and Sitka spruce under field test conditions.

This required :

i) An investigation of the most appropriate delivery system for *Trichoderma*;

ii) Assessment of *Trichoderma* colonisation and survival;

iii) Measurement of permeability enhancement in Scots pine and Sitka spruce sapwood and heartwood in treated logs;

iv) Assess the efficiency of creosote retention and penetration in treated logs.

Chapter 2. Degradative Extra cellular Enzyme Production by Selected *Trichoderma* Isolates.

2.1 CULTURING OF FUNGAL ISOLATES.

2.1.1 Introduction.

Collections of fungi are usually preserved on a growth medium that will allow the organism to survive in a dormant form until introduced to favourable conditions for growth. The Scottish Institute for Wood Technology (SIWT) holds a collection of cultures of various wood associated fungi. These cultures are preserved using either simple refrigeration, freeze drying, or storage under sterile mineral oil or on sterile dried silica gel. Fungi stored in such a way can normally be sub-cultured from stock and exhibit the same growth characteristics as the original cultures.

2.1.2 Methods

Sterile agar plates were prepared using 3% malt extract agar (Oxoid) autoclaved at 121°C for 20 minutes. Each of these plates were then inoculated with one of 47 *Trichoderma* isolates (Table 2.1) and incubated at appropriate optimum temperature for each isolate (either 22 or 25°C). After successful colonisation of the plates, the isolates were sub-cultured onto malt extract agar slopes for storage.

Slopes were prepared by adding 10mls of 3% malt extract agar to universal bottles and autoclaving at 121°C for 20 minutes. After sterilisation, universals were placed on racks to produce sloped agar on setting. These slopes were then inoculated with a 8mm core from one of the separate *Trichoderma* isolates and incubated at the appropriate optimum temperature for each (either 22 or 25°C; Table 2.1). Once colonised these slopes were returned to the culture collection.

***Trichoderma* isolates with optimum growth temperature of 25 ^{o}C**	***Trichoderma* isolates with optimum growth temperature of 22 ^{o}C**
Trichoderma aureoviride SIWT.1	*Trichoderma viride* SIWT. 11
T. harzianum IMI 206040	*T. viride* SIWT. 14
T. polysporum IMI 206039	*T. viride* SIWT. 24
T. viride IMI 24039	*T. viride* SIWT. 28
T. viride IMI 49791	*T. viride* SIWT. 30
Trichoderma CCA sample	*Trichoderma* SIWT. 38
Trichoderma FYT strain	*T. viride* SIWT. 40
T. viride IMI 335517	*T. viride* SIWT. 43
T. harzianum IMI 335518	*T. viride* SIWT. 53
T. pseudokoningii SIWT. 22	*T. viride* SIWT. 60
T. harzianum SIWT. 25	*T. viride* SIWT. 67
T. pseudokoningii SIWT. 33	*T. viride* SIWT. 70
T. pseudokoningii SIWT. 51	*T. viride* SIWT. 90
T. pseudokoningii SIWT. 55	*T. viride* SIWT. 100
T. pseudokoningii SIWT. 64	*T. viride* SIWT. 110
Trichoderma SIWT. 140	*T. hamatum* SIWT. 150
Trichoderma (ASH)	*Trichoderma* SIWT. 170
T. citrinoviride IMI 335519	*Trichoderma* SIWT. 190
T. saturnisporum IMI14685	*T. polysporum* SIWT. 220
T. longibrachiatum IMI 536408	*T. hamatum* SIWT. 4
T. reesei IMI 192656ii	*T. polysporum* SIWT. 13
T. saturnisprorum SIWT. 142	*T. saturnisporum* SIWT. 91
	T. hamatum SIWT. 44
	T. polysporum SIWT. 200
	T. saturnisporum SIWT. 69

Table 2.1 Optimum growth temperatures of *Trichoderma* isolates held in the Scottish Institute for Wood Technology's (SIWT.) culture collection.

2.2 GROWTH RATE EXPERIMENTS.

2.2.1 Introduction.

Trichoderma like other fungi have been shown to respond to different growth conditions (Eastburn and Butler, 1991). Morton and Eggins (1977) showed the effects of constant, altering and fluctuating temperatures on the growth of some wood inhabiting fungi, where generally lower temperatures reduced the growth rate of the organism. If *Trichoderma* isolates are to improve wood permeability under field conditions they should not be sensitive to the temperatures likely to be experienced under these conditions. The aim of this experiment was to investigate the growth of stock *Trichoderma* cultures over a range of different temperatures.

2.2.2 Methods.

A low nutrient medium with a Carbon : Nitrogen ratio similar to wood, based on that used by Huttermann and Volger (1973) was prepared for this study (table 2.2).

Chemical	Quantity/Litre
Glucose	5.000 g
L-Asparagine	0.013g
NH_4NO_3	0.008g
KH_2PO_4	1.000g
$MgSO_4$ (anhydrous)	0.300g
KCl	0.500g
$FeSO_4$ (anhydrous)	0.010g
$Mn(CH_3COO)_2x4H_2O$	0.008g
$Zn(NO_3)_2x6H_2O$	0.002g
$Ca(NO_3)_2x4H_2O$	0.050g
$CuSO_4$ (anhydrous)	0.002g

Table 2.2 Recipe for minimal medium (after Hutterman and Volger, 1973), NB the pH of the media was the checked and if necessary adjusted to pH 5.5.

All constituents were dissolved in 1000 ml distilled water and sterilised by autoclaving at 121°C for 20 minutes. If solid agar plates were required then 1% purified agar was added to the medium prior to sterilisation. Twenty millilitres of sterile medium was transferred into petri dishes and four sets of agar plates were inoculated with each of the 47 different *Trichoderma* isolates (3 replicates of each isolate) and incubated at either 25°C , 22°C, 14°C or 4 °C. Growth rates of each isolate were determined by measuring hyphal extension across the agar plates on a daily basis until the organism had reached the edge of the plate.

2.2.3 Results

The different growth rates observed from incubating the different *Trichoderma* isolates at different temperatures can be seen in tables 2.3 a and b. The results show a general decline in the growth rate as the temperature is decreased.

Isolate	Incubation Temperature			
	25°C	**22°C**	**14°C**	**4°C**
Trichoderma aureoviride SIWT.1	6.75	7.75	2.50	0.62
T. harzianum IMI 206040	8.75	8.62	1.00	0.75
T. polysporum IMI 206039	5.88	7.12	1.25	0.56
T. viride IMI 24039	10.00	10.00	1.50	x
T. viride IMI 49791	10.00	10.00	3.25	x
Trichoderma CCA sample	10.00	10.00	2.50	0.38
Trichoderma FYT strain	8.62	6.12	.50	0.62
T. viride IMI 335517	8.12	10.00	3.75	0.38
T. harzianum IMI 335518	6.38	7.50	2.25	1.75
T. pseudokoningii S.I.W.T 22	12.00	10.00	3.00	0.75
T. harzianum S.I.W.T 25	10.00	10.00	3.75	0.56
T. pseudokoningii S.I.W.T 33	10.00	10.00	1.75	1.06
T. pseudokoningii S.I.W.T 51	10.00	10.00	2.25	0.19
T. pseudokoningii S.I.W.T 55	10.00	10.00	2.50	0.56
T. pseudokoningii S.I.W.T 64	10.00	10.00	2.50	0.75
Trichoderma S.I.W.T 140	10.00	10.00	2.50	0.25
Trichoderma (ASH)	11.00	10.00	2.50	0.43
T. citrinoviride IMI 335519	13.25	10.00	3.00	0.82
T. saturnisporum IMI14685	10.00	10.00	1.75	x
T. longibrachiatum IMI 536408	13.50	10.00	3.25	0.19
T. reesei IMI 192656ii	8.00	5.50	1.00	0.19
T. saturnisprorum S.I.W.T 142	10.00	10.00	44.0	1.94

Table 2.3a Mean growth rate (mm/day) of different *Trichoderma* isolates under various incubation temperatures. Note. "x" represents no growth observed.

Isolate	Incubation Temperature			
	25°C	22°C	14°C	4°C
Trichoderma viride SIWT 11	5.62	4.50	3.50	1.69
T. viride S.I.W.T 14	1.50	1.25	0.75	0.50
T. viride S.I.W.T 24	6.38	2.12	3.50	1.88
T. viride S.I.W.T 28	10.00	6.75	4.25	1.43
T. viride S.I.W.T 30	10.00	7.50	4.25	1.75
Trichoderma S.I.W.T 38	10.00	7.25	3.25	2.28
T. viride S.I.W.T 40	10.00	6.25	3.75	1.06
T. viride S.I.W.T 43	8.12	10.00	3.75	1.75
T. viride S.I.W.T 53	6.50	8.38	6.75	1.75
T. viride S.I.W.T 60	3.50	2.50	1.25	0.75
T. viride S.I.W.T 67	8.12	8.12	5.25	1.62
T. viride S.I.W.T 70	7.50	5.00	1.25	1.50
T. viride S.I.W.T 90	8.25	6.25	4.75	1.88
T. viride S.I.W.T 100	7.25	6.12	4.00	1.62
T. viride S.I.W.T 110	8.75	7.66	3.00	2.00
T. hamatum S.I.W.T 150	8.25	7.75	5.00	1.31
Trichoderma S.I.W.T 170	6.50	6.25	3.75	1.69
Trichoderma S.I.W.T 190	3.75	3.75	2.25	1.69
T. polysporum S.I.W.T 220	4.00	3.38	2.00	2.06
T. hamatum S.I.W.T 4	9.00	3.62	4.75	2.35
T. polysporum S.I.W.T 13	7.50	5.38	3.75	2.75
T. saturnisporum S.I.W.T 91	8.00	7.50	4.75	1.31
T. hamatum S.I.W.T 44	10.00	10.00	5.00	1.00
T. polysporum S.I.W.T 200	3.25	4.12	2.25	1.50
T. saturnisporum S.I.W.T 69	10.75	10.00	3.50	1.12

Table 2.3b Mean growth rate (mm/day) of different *Trichoderma* isolates under various incubation temperatures.

Most of the *Trichoderma* isolates tested showed significant growth at all of the temperatures tested. Only four isolates out of the 47 tested showed no growth at the lowest incubation temperature. Not all isolates showed the maximum growth at their expected optimal temperature. These differences were probably due to the growth medium used. The previously determined optima were established on malt extract media which will provide the organisms with different growth conditions, nutrients and stimuli. However at 22 and 25°C the growth rates were significantly faster than at lower incubation temperatures.

2.3 EXTRA CELLULAR ENZYME PRODUCTION BY *TRICHODERMA* ISOLATES.

2.3.1 Introduction.

Microorganisms are responsible for the recycling of much of the organic material in the environment. The constituent materials of dead organisms are liberated and utilised by decomposer microorganisms. These constituents are found in two forms; low molecular weight, water-soluble, materials that are readily absorbed, or more complex macromolecules. In plants these include cellulose, hemi-cellulose, lignin, pectin and starch. Some fungi utilise these compounds by secreting enzymes that break down these macromolecules in the environment and the organisms then assimilate the low-molecular weight products (Priest, 1984)

Such degradative enzymes can be produced constitutively where enzyme synthesis is constant irrespective of the presence of substrate. More commonly enzyme synthesis is induced by the presence of substrate or product in the growth medium and are present only in small quantities in the absence of the substrate. Generally when the substrate or derivatives are present in the medium there is a dramatic increase in the rate of enzyme synthesis for the catabolism of that substrate. The production of such enzymes can however also be repressed. Repression of

enzymes can take two forms; 1) if the product of a particular enzyme inhibits its synthesis then this repression is specific for the particular enzyme and is often referred to as end-product repression; 2) the repression of enzymes involved in the catabolism of complex carbon sources by readily utilisable carbon sources (e.g. glucose) is termed catabolite repression. (Priest,1984)
Catabolite repression is the repression of inducible or constitutive enzyme synthesis and occurs in most microorganisms growing on any readily utilised carbon source.

Extra cellular enzyme production by fungi has a major role in the cycling of materials in the environment e.g. in the carbon cycle. This enzyme production has been commercially exploited for bio-remediation of waste products e.g. paper pulp waste, as well as the purification of products prior to usage e.g. clearing of fruit fermentation (Priest, 1984).

Wood permeability is reliant on the condition of the constituent fibres and inter-connecting apertures and can be altered by the action of enzymes on the wood (King and Eggins, 1973). Inter-tracheid pit membranes being mainly composed of pectin can be de-aspirated by the action of pectinase. Stored starch in ray fibres will radically affect the permeability of the timber in a radial direction, and the action of amylase may convert this starch to readily utilisable simple sugar components. Pit-membranes are held in place by cellulose microfibrils which if degraded may prevent or reverse pit aspiration.

The aims of the following experiments were to investigate the production and activity of extra cellular pectinase, amylase and cellulase by *Trichoderma* isolates.

2.3.2 Pectinase Production and Activity by *Trichoderma* isolates.

2.3.2.1 Qualitative Assay for Pectinase Production by *Trichoderma* isolates.

A qualitative assay was devised to demonstrate that *Trichoderma* isolates could produce extra-cellular pectinase. The assay involved the use of two different medium, one containing sterile distilled water and 6% pectin (w/v), the other containing sterile low nutrient broth (section 2.2.2) and 6% pectin (w/v). Five ml of the medium were then asceptically transferred into sterile test-tubes. Two tubes of each medium type were inoculated with the *Trichoderma* isolates to be screened in the assay, two tubes of each medium were left uninoculated to act as controls. Positive controls were also included by adding a commercially available pectinase (Pectinex 1XL, Novo Nordisk Ferment Ltd) to replicate tubes of the medium. A positive result was recorded if the medium inoculated with *Trichoderma* or pectinase was liquefied and the negative controls remained solid after 5 days incubation at 22 or 25^{o}C .

On the basis of the results of the qualitative assay only those *Trichoderma* isolates producing liquefaction of either medium were subsequently screened quantitatively.

2.3.2.2. Quantitative Assay for Extra cellular Pectinase Activity.

After initial qualitative pectinase screening, 35 of 47 isolates produced a positive result. These isolates were assayed using a variation of the method of Sandhu and Kalra (1982). Isolates were subcultured by adding a single 8mm core of the fungus into flasks containing low nutrient broth (section 2.2.2) and 1% pectin. Two types of medium were used for this screening. One contained the normal 0.5% glucose, while the other contained no sugar to determine whether glucose content had induced catabolite repression. Isolates were incubated

at their optimum temperature for 4 days before being filtered through a 0.45μm filter. The filtrates were then frozen at -20°C until assayed.

The assay consisted of the filtrate being incubated in buffered pectin solution for 2 hours and the increase in flow rate being used as a measure of pectinase activity. Two ml of the defrosted filtrate was combined with 10 ml of 1 % aqueous solution of pectin (w/v), 3 ml citrate buffer (0.05M, pH 4.8) and 2 ml distilled water and mixed thoroughly. The flow rate of the pectin solution plus filtrate was measured immediately by timing the running of 5 ml of the solution through a 5ml grade "A" pipette prior to incubation. This process was done in duplicate to verify the initial flow time of the solution through pipette and control samples of deactivated filtrate were also included in the assay. The samples were then incubated for 2 hrs at 30°C after which the flow time of 5 ml of the solution was again measured in duplicate. From these results it was possible to determine the increase in flow rate of the solution after incubation with the enzyme and hence indirectly determine which isolates produced the largest amounts of pectinase under these experimental conditions.

Pectinase activity was calculated as a mean % loss in viscosity as follows:

$$\frac{Vo-Vt}{Vo-Vw} \times 100$$

Where:

Vo = flow time 0 hour.

Vt = flow time after 2 hours incubation.

Vw = flow time of distilled water.

2.3.2.3 Results of Pectinase Assays

The results of the qualitative pectinase assay can be seen in table 2.4.a and b:

***Trichoderma* isolate**	**Aqueous Pectin solution**	**Pectin and Minimal Medium**
Trichoderma aureoviride SIWT.1	✓	✓
T. harzianum IMI 206040	✓	X
T. polysporum IMI 206039	✓	X
T. viride IMI 24039	✓	X
T. viride IMI 49791	✓	X
Trichoderma CCA sample	✓	X
Trichoderma FYT strain	✓	X
T. viride IMI 335517	✓	X
T. harzianum IMI 335518	✓	X
T. pseudokoningii SIWT. 22	✓	X
T. harzianum SIWT. 25	✓	X
T. pseudokoningii SIWT. 33	✓	X
T. pseudokoningii SIWT. 51	✓	X
T. pseudokoningii SIWT. 55	✓	X
T. pseudokoningii SIWT. 64	✓	X
Trichoderma SIWT. 140	✓	X
Trichoderma (ASH)	✓	X
T. citrinoviride IMI 335519	✓	X
T. saturnisporum IMI14685	✓	X
T. longibrachiatum IMI 536408	X	X
T. reesei IMI 192656ii	X	X
T. saturnisprorum SIWT. 142	X	X

Table 2.4.a Qualitative results of pectinase assay for *Trichoderma* isolates incubated at 25°C. N.B. "✓" represents both tubes showing liquefaction of medium. "**X**" represents medium showing no enzyme activity.

Trichoderma isolate	Aqueous Pectin solution	Pectin and Minimal Medium
Trichoderma viride SIWT. 11	✓	X
T. viride SIWT. 14	✓	X
T. viride SIWT. 24	✓	X
T. viride SIWT. 28	X	X
T. viride SIWT. 30	✓	X
Trichoderma SIWT. 38	✓	X
T. viride SIWT. 40	X	X
T. viride SIWT. 43	✓	X
T. viride SIWT. 53	✓	X
T. viride SIWT. 60	X	X
T. viride SIWT. 67	X	X
T. viride SIWT. 70	✓	X
T. viride SIWT. 90	✓	X
T. viride SIWT. 100	X	X
T. viride SIWT. 110	X	X
T. hamatum SIWT. 150	X	X
Trichoderma SIWT. 170	✓	X
Trichoderma SIWT. 190	X	X
T. polysporum SIWT. 220	✓	X
T. hamatum SIWT. 4	✓	X
T. polysporum SIWT. 13	✓	✓
T. saturnisporum SIWT. 91	✓	X
T. hamatum SIWT. 44	✓	X
T. polysporum SIWT. 200	X	X
T. saturnisporum SIWT. 69	✓	X

Table 2.4.b Qualitative results of pectinase assay for *Trichoderma* isolates incubated at 22°C. N.B. "✓" represents both tubes showing liquefaction of medium by enzyme production. "**X**" represents medium showing no enzyme activity.

The results in table 2.4 a and b show that not all *Trichoderma* isolates show pectinase activity. The inclusion of minimal medium has inhibited the production of pectinase by most isolates and demonstrates that catabolite repression of the pectinase enzymes has occurred in all isolates except *Trichoderma aureoviride* SIWT.1 and *T. polysporum* SIWT. 13.

The results of quantitative pectinase assays can be seen in tables 2.5 a and b:

***Trichoderma* isolate**	**Pectinase activity in minimal medium excluding glucose**	**Pectinase activity in minimal medium including glucose**
Trichoderma aureoviride SIWT.1	71.7	16.7
T. harzianum IMI 206040	69.2	0
T. polysporum IMI 206039	84.0	14.3
T. viride IMI 24039	53.33	15.8
T. viride IMI 49791	68.33	6.7
Trichoderma CCA sample	50.00	5.6
Trichoderma FYT strain	56.25	0
T. viride IMI 335517	20.0	14.2
T. harzianum IMI 335518	70.0	17.6
T. pseudokoningii SIWT. 22	57.0	0
T. harzianum SIWT. 25	60.0	13.3
T. pseudokoningii SIWT. 33	61.7	6.25
T. pseudokoningii SIWT. 51	33.3	0
T. pseudokoningii SIWT. 55	46.4	0
T. pseudokoningii SIWT. 64	65.0	23.5
Trichoderma SIWT. 140	50.0	6.25
Trichoderma (ASH)	47.1	5.9
T. citrinoviride IMI 335519	32.5	0
T. saturnisporum IMI14685	35.0	12.5

Table 2.5a Quantitative pectinase activity results for *Trichoderma* isolates incubated at 25°C, figures represent the mean % loss in viscosity of a pectin solution after incubation with the filtrate for 2 hours at 30°C.

Trichoderma isolate	Pectinase activity in minimal medium excluding glucose	Pectinase activity in minimal medium including glucose
Trichoderma viride SIWT. 11	65.0	15.8
T. viride SIWT. 14	8.2	0
T. viride SIWT. 24	43.7	11.1
T. viride SIWT. 30	28	6.7
Trichoderma SIWT. 38	18.7	0
T. viride SIWT. 43	23.3	6.7
T. viride SIWT. 53	18.0	11.1
T. viride SIWT. 70	86.7	12.5
T. viride SIWT. 90	38.9	6.7
Trichoderma SIWT. 170	23.0	13.3
T. polysporum SIWT. 220	22.0	6.25
T. hamatum SIWT. 4	33.0	0
T. polysporum SIWT. 13	24.0	0
T. saturnisporum SIWT. 91	21.0	7.1
T. hamatum SIWT. 44	25.0	25.0
T. saturnisporum SIWT. 69	50.0	6.7

Table 2.5 b Quantitative pectinase activity results for *Trichoderma* isolates incubated at 22°C, figures represent the mean % loss in viscosity of a pectin solution after incubation with the filtrate for 2 hours at 30°C. Little difference in % loss in viscosity was recorded in the two duplicate samples for each isolate (see Appendix 1).

The results in tables 2.5 a and b show that in nearly every case the pectinase activity observed is reduced when the isolates are grown in the presence of glucose. This is indicative of catabolite repression. The quantitative assay highlights the variable activities of the pectinase produced by the different *Trichoderma* isolates.

2.3.2.4. Discussion

Pectin is a complex carbohydrate, which has long been known to be widely distributed in the vegetable kingdom (Trehan and Ahmad, 1947). Pectic substances are known to occur in all plant tissues (Sharma and Kumar, 1979), their location being mostly in the primary walls and pit membranes. Bauch *et al* (1968) found that the main locations of pectin in some pines was in the middle lamella and torus of bordered pit membranes.

Poor permeability is often the limiting factor for preservative treatment and is dependent on the condition of bordered pit membranes connecting tracheids. Biological agents capable of attacking pectic substances have been shown to improve the permeability of the timber to subsequent preservative treatment and may have little effect on strength. (Bauch *et al*, 1970). Several authors have shown that pectinase will improve the permeability and treatability of different timber species (Nicholas and Thomas, 1968 b; Bauch *et al*, 1970; Fogarty, 1973; Ohkoshi *et al* 1987; Militz, 1993 a; Militz, 1993 b). If *Trichoderma* isolates are to improve the permeability of Sitka spruce (*Picea sitchensis*) and Scots pine (*Pinus sylvestris*) then by implication the isolates used will have to be significant producers of pectinase.

From the results it can be seen that the enzyme activity produced under these experimental conditions varied between isolates. In the first experiment only 35 of the original 47 isolates produced pectinase in the qualitative assay. The quantitative assay confirmed that these 35 isolates produced significant amounts of pectinase activity. The production of pectinase was inhibited by the inclusion of glucose in the growth medium and appears indicative of catabolite repression of the enzyme.

The repression of pectinase may have important implications when the organism grows through wood. Green wood contains a complex mixture of non-structural photosynthetic

products in both mobile and stored forms which may inhibit pectinase production in the first instance. Once these simple carbohydrates are exhausted by any colonising organism pectinase synthesis may increase.

Trichoderma isolates have been identified as possible sources of commercial pectinase (Sharma and Kumar, 1979 ; Archer and Wood, 1994) but this will obviously be dependent on the growth medium, culture conditions and isolate used. Pectin is a complex carbohydrate and if it is to be broken down completely then different forms of pectinase must be produced by decomposing organisms (Archer and Wood, 1994) e.g. *Aspergillus niger* can produce 5 different endopolygalacturonases and these may be secreted in response to different environmental conditions (Kester and Visser , 1990). Cruickshank and Wade (1980) used poly-acyrlamide gels to detect three different types of pectinase namely; pectin esterase, polygalacturonase and pectin lyase from various organisms including *Aspergillus niger*, *Penicillium atrovenetum* and *Scleretonia sclerotiorum*. However the screening method used in this study measured only gross pectinase activity on a buffered pectin solution and did not differentiate between the different types of pectinase. It could be hypothesised that if *Trichoderma* isolates produced a range of different pectinase enzymes similar to *Aspergillus niger* and *Penicillium atrovenetum* this may provide one explanation why some activity was observed in medium containing glucose as only some and not all of the pectinase enzymes were subject to repression in the culture conditions.

The production/ activity of pectinase in minimal medium was also significantly lower for the tested isolates than that seen with commercially produced pectinase e.g. the flow rate after a commercial enzyme incubation initial flow time of a 1:10000 dilution was 21.5 seconds, and after incubation this dropped to 14.5 seconds. Where as the flow rates for isolate *Trichoderma aureoviride* SIWT.1 the neat filtrate of which had an initial flow time of 24.4 seconds which dropped to 11.06 seconds. The higher activity of the commercial enzyme is

not altogether surprising as isolates used for commercial pectinase production will be specially selected for the process and enzymes may be concentrated. The low nutrient medium used for the assay was a minimal medium with a C:N ratio similar to wood and commercial isolates would normally produce pectinase under ideal conditions for the isolates (Priest, 1984) and not under the relatively spartan conditions experienced with minimal medium.

2.3.3 Production and activity of Amylase enzymes from *Trichoderma* isolates.

2.3.3.1 Qualitative Assay for Amylase Production.

Initial studies were required to show that *Trichoderma* isolates would produce extra-cellular amylase. Sterile agar plates containing 3% malt extract agar (Oxoid) and 0.5% soluble starch were prepared and inoculated with each of the 47 *Trichoderma* isolates. The plates were then incubated at the optimum temperature for the isolates (either 22 or 25^{o}C) until the fungi had nearly reached the edge of the plate. The plates were then flooded with iodine. These plates were scored by determining whether the area beneath the colony did not stain because of the conversion of starch to reducing sugars by amylase enzymes. This experiment indicated that this simple methodology could be used to qualitatively assess amylase production.

The experiment was repeated with minimal medium (section 2.2.2) replacing malt extract agar medium. These plates were incubated at the optimum temperature for the isolates (either 22 or 25^{o}C) and then flooded with iodine. The iodine penetrated the hyphal mat and stained the starch that remained in the agar at the end of the incubation. The zone of clearing around the *Trichoderma* colonies measured and only those isolates showing the largest zone of clearing ($\geq$23mm) were subsequently assayed quantitatively.

2.3.3.2. Quantitative Assay for Extracellular Amylase Activity.

After initial screening the 25 isolates showing the largest cleared zones, were subsequently assayed using a variation on the method used by Oso (1979). Selected isolates were grown in 2 different minimal medium broths (section 2.2.2) plus 0.5% soluble starch. One medium contained 0.5% glucose, the other contained no added glucose. The inclusion of the glucose in the growth medium was to determine if catabolite repression occurred. After 4 days incubation at the optimum temperature for each isolate (22 or 25°C) the cultures were harvested by filtration through a 0.45μm filter. The resulting filtrate was then used in a quantitative assay for amylase activity.

A solution of 1% soluble starch in 0.02M phosphate buffer (pH 6.9) was prepared and 1 ml of enzyme filtrate was added to 1 ml of buffered starch solution and incubated at 45°C for 1 hour. The amount of reducing sugar released was measured by adding 1 ml of the incubated enzyme-starch solution to 1 ml dintrosalicylic-acid (DNSA) and incubated in a covered tube at 100°C for 10 minutes to allow colour to develop before adding 10 ml of distilled water. The absorbance of the resulting developed solution was measured at 540 nm in a Perkin Elmer spectrophotometer. Each culture filtrate was assayed in duplicate. A standard curve for this DNSA assay was determined by assaying known standard aqueous solutions of maltose i.e. 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 and 6 mg ml^{-1} as above.
Enzyme filtrates contained residual reducing sugars and hence this DNSA assay was replicated prior to incubation with the buffered starch solution. Corrected values for each filtrate were then calculated by subtracting this value from readings recorded after incubation with starch solutions.

2.3.3.3 Results.

The results of the qualitative assays can be seen in tables 2.6a and b:

Trichoderma isolate	Malt Extract	Minimal medium containing glucose
Trichoderma aureoviride SIWT.1	✓	✓ (23mm)
T. harzianum IMI 206040	✓	✓ (24mm)
T. polysporum IMI 206039	✓	✓ (27mm)
T. viride IMI 24039	✓	✓ (22mm)
T. viride IMI 49791	✓	**X** (0mm)
Trichoderma CCA sample	✓	✓ (22mm)
Trichoderma FYT strain	✓	✓ (34mm)
T. viride IMI 335517	✓	**X** (0mm)
T. harzianum IMI 335518	✓	✓ (24mm)
T. pseudokoningii SIWT. 22	✓	✓ (23mm)
T. harzianum SIWT. 25	✓	✓ (23mm)
T. pseudokoningii SIWT. 33	✓	✓ (28mm)
T. pseudokoningii SIWT. 51	✓	✓ (33mm)
T. pseudokoningii SIWT. 55	✓	✓ (25mm)
T. pseudokoningii SIWT. 64	✓	✓ (30mm)
Trichoderma SIWT. 140	✓	✓ (32mm)
Trichoderma (ASH)	✓	✓ (28mm)
T. citrinoviride IMI 335519	✓	✓ (25mm)
T. saturnisporum IMI14685	✓	✓ (30mm)
T. longibrachiatum IMI 536408	✓	✓ (24mm)
T. reesei IMI 192656ii	✓	✓ (30mm)
T. saturnisprorum SIWT. 142	✓	✓ (20mm)

Table 2.6 a Qualitative results of amylase assay for *Trichoderma* isolates incubated at 25°C. N.B. "✓" represents presence of clear area when stained with iodine. "**X**" represents no enzyme activity (figures in parenthesis indicate diameter of zone of clearing around colony).

***Trichoderma* isolate**	**Malt Extract**	**Minimal medium containing glucose**
Trichoderma viride SIWT. 11	✓	**X** (0mm)
T. viride SIWT. 14	✓	**X** (0mm)
T. viride SIWT. 24	✓	✓ (21mm)
T. viride SIWT. 28	✓	✓ (18mm)
T. viride SIWT. 30	✓	✓ (18mm)
Trichoderma SIWT. 38	✓	**X** (0mm)
T. viride SIWT. 40	✓	✓ (20mm)
T. viride SIWT. 43	✓	**X** (0mm)
T. viride SIWT. 53	✓	✓ (27mm)
T. viride SIWT. 60	✓	✓ (23mm)
T. viride SIWT. 67	✓	✓ (18mm)
T. viride SIWT. 70	✓	✓ (26mm)
T. viride SIWT. 90	✓	✓ (25mm)
T. viride SIWT. 100	✓	✓ (24mm)
T. viride SIWT. 110	✓	**X** (0mm)
T. hamatum SIWT. 150	✓	✓ (19mm)
Trichoderma SIWT. 170	✓	✓ (25mm)
Trichoderma SIWT. 190	✓	✓ (15mm)
T. polysporum SIWT. 220	✓	✓ (28mm)
T. hamatum SIWT. 4	✓	✓ (12mm)
T. polysporum SIWT. 13	✓	✓ (15mm)
T. saturnisporum SIWT. 91	✓	✓ (23mm)
T. hamatum SIWT. 44	✓	**X** (0mm)
T. polysporum SIWT. 200	✓	✓ (17mm)
T. saturnisporum SIWT. 69	✓	✓ (22mm)

Table 2.6 b Qualitative results of amylase assay for *Trichoderma* isolates incubated at 22°C. N.B. "✓" represents presence of clear area when stained with iodine. "**X**" represents no enzyme activity (figures in parenthesis indicate diameter of zone of clearing around colony).

The results in tables 2.6 a and b show that when grown on malt extract agar containing starch all isolates produced amylase. When this was repeated on a minimal medium most isolates continued to produce amylase however 8 isolates showed no production even after 7 days incubation. The size of the zone of clearing is an indication of the production of amylase hence only those isolates showing the greatest zones of clearing were subsequently screened quantitatively i.e. a zone ≥ 23 mm. This reduced the number of isolates assayed quantitatively to 25.

The results from the DNSA assay were calculated against a standard curve (figure 2.1.) using known standard concentrations of maltose.

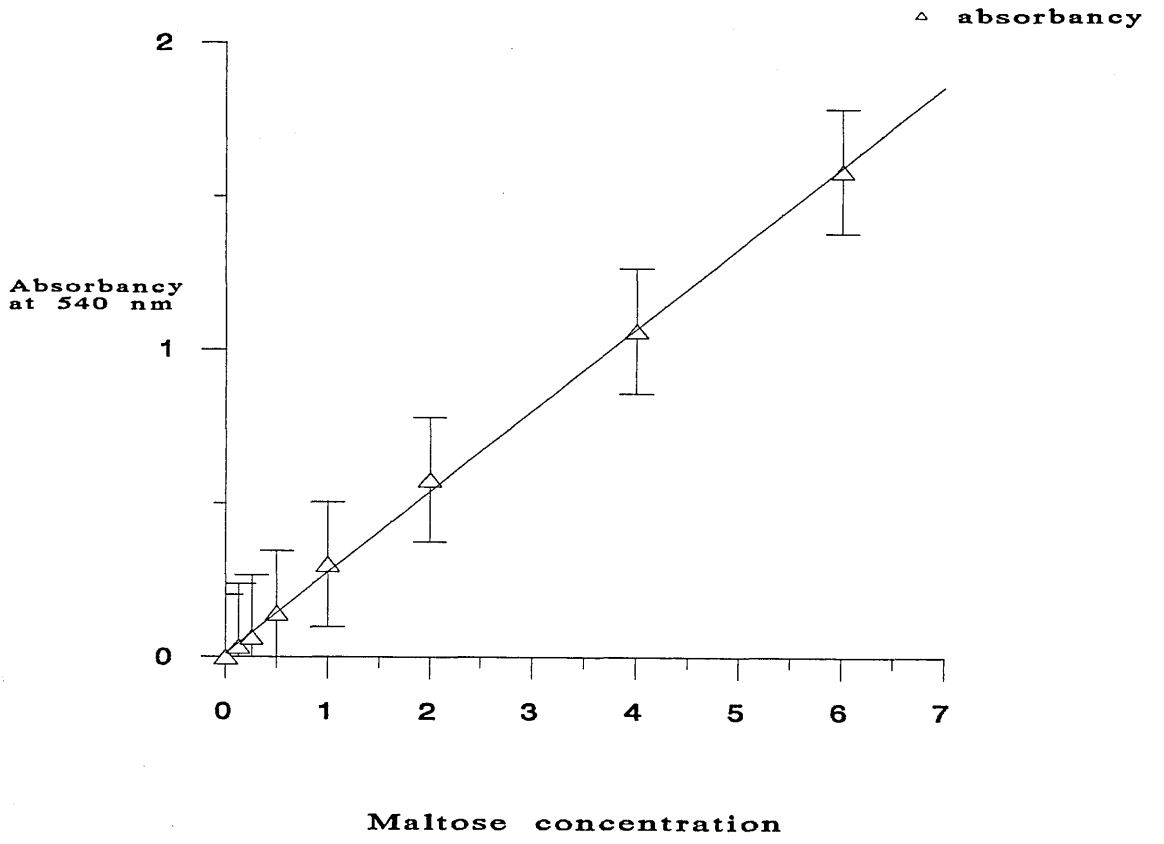


Figure 2.1 Standard curve for calculation of reducing sugar concentrations (mg/ml) in assays of reducing sugar content. N.B. Error bars were calculated as the standard error of the data set.

Using the equation ($Y = 0.26x$) calculated from the graph , the concentrations of reducing sugar in the samples were determined. The amounts of sugars released after incubation with the culture filtrates can be seen in table 2.7. after subtraction of residual sugar content of the filtrate (section 2.3.3.2)

***Trichoderma* isolate**	**Reducing sugar released from filtrate of cultures with added glucose (mg/ml)**	**Reducing sugar released from filtrate of cultures with no added glucose (mg/ml)**
Trichoderma aureoviride SIWT.1	0	0.50
T. harzianum IMI 206040	0	0.48
T. polysporum IMI 206039	0	1.38
Trichoderma FYT strain	0.18	1.10
T. harzianum IMI 335518	0	0.07
T. pseudokoningii SIWT. 22	0	1.50
T. harzianum SIWT. 25	0.67	0.51
T. pseudokoningii SIWT. 33	0	0.37
T. pseudokoningii SIWT. 51	0	1.46
T. pseudokoningii SIWT. 55	0	0.59
T. pseudokoningii SIWT. 64	0	0.85
Trichoderma SIWT. 140	0	0.83
Trichoderma (ASH)	0	1.25
T. citrinoviride IMI 335519	0	0.45
T. saturnisporum IMI14685	0	0.33
T. longibrachiatum IMI 536408	0.26	0.10
T. reesei IMI 192656ii	0.58	1.30
T. viride SIWT. 53	0	0.55
T. viride SIWT. 60	0	0.39
T. viride SIWT. 70	0	0.99
T. viride SIWT. 90	0	1.12
T. viride SIWT. 100	0	0.58
Trichoderma SIWT. 170	0.25	3.76
T. polysporum SIWT. 220	0.13	0.20
T. saturnisporum SIWT. 91	0	0.99

Table 2.7 Amylase activity of filtrates of selected *Trichoderma* isolates. N.B. figures represent average amount (mg/ml) of reducing sugar released after 1hrs incubation at 45^{o}C .

As with pectinase *Trichoderma* isolates showed variability in the levels of enzyme activity produced. In most cases medium containing glucose showed lower amylase activity which is due to catabolite repression. Exceptions being *Trichoderma harzianum* SIWT. 25 and *T. longibrachiatum* IMI 536408 which gave higher amylase production in medium with glucose present. Little difference was observed in the amount of reducing sugar released from duplicate samples (see appendix 2).

2.3.3.4 Discussion.

The results in tables 2.6 a and b show that when grown on malt extract agar containing starch all of the tested isolates produced significant amounts of amylase. When minimal medium replaced the malt extract and the experiment was repeated 8 isolates failed to produce any amylase. The low nutrient medium has prevented these isolates from producing amylase by inhibiting the production of the enzyme. This may simply be due to nutrient stress of the organism or the fact that the glucose in the minimal medium has caused greater repression of the amylase activity.

Those isolates producing amylase showed differences in the activity of enzyme produced (table 2.7). Differences may be expected as these isolates will be adapted to their own niche in nature and hence will not necessarily be expected to produce similar amounts of the assayed enzymes.

When the results of the quantitative assays were investigated, most isolates showed catabolite repression when grown on the medium containing glucose. When grown on minimal medium with no added glucose the assayed enzyme activity is greater which is most likely due to greater production of the enzyme on this medium.

Starch in wood is normally only present in small amounts (Farmer, 1967). The production of amylase is vital for the conversion of stored starch into component reducing sugars i.e. maltose or glucose (Priest, 1984) which can be utilised for fungal or other microbial growth.

In many hardwoods carbohydrates are the most common form of storage product whereas "fats" or "resins" are the most common storage products in softwoods (Hillis, 1987). Indeed when present, the distribution of starch can be localised within the timber and generally decreases with height and towards the heartwood boundary. Hence the production of amylase by the *Trichoderma* isolates may only have small implications in the improvement of the permeability of *Pinus sylvestris* and *Picea sitchensis.*

2.3.4 Cellulase Production and Activity by *Trichoderma* isolates.

2.3.4.1 Qualitative Cellulase Production

Two different low nutrient agar (see section 2.2.2) medium were prepared, one containing (0.5% w/v) glucose the other no glucose. Cellulose azure was sterilised in an 160^{o}C oven for $1^{1}/_{2}$ hours. The sterilised azure was sprinkled on top of the growth medium prior to inoculation with the *Trichoderma* isolates. The tubes were then incubated at the appropriate optimum temperature for the *Trichoderma* isolates for 7 days. After incubation the tubes were inspected for dye release from the cellulose azure. Although good growth of the organisms was observed, no dye was released from the azure. Since *Trichoderma* isolates have historically been used to produce commercial cellulase it was highly unlikely that all of the tested isolates would be unable to produce cellulase. It was therefore necessary to screen all of the isolates quantitatively.

2.3.4.2 Quantitative Assay for Extra cellular Cellulase.

As with previous enzyme assays the *Trichoderma* isolates were cultured on low nutrient broth (section 2.2.2) with and without 0.5% glucose, and with carboxy-methyl cellulose (CMC) as the cellulose source (1%w/v). After 4 days incubation at appropriate temperatures the cultures were filtered though a 0.45µm filter and the filtrate retained for use in the enzyme assay based on that used by Greaves (1971).

Citrate buffer (0.05M) was prepared by dissolving 14.7g sodium citrate in 1 litre distilled water and the pH adjusted to 4.5. A solution of CMC was prepared by adding 6g of air dry CMC to 500 ml of buffer citrate. This was agitated using a waring blender until the solution was clear and any lumps in the solution were removed by filtration. 5 ml of a 1% merthiolate solution was added to the CMC solution.

The assay was prepared by placing 10 ml of CMC solution into universal bottles, 2 ml of enzyme filtrate was then added to the solution and the flask shaken to mix the solutions. The flow time of the solutions was measured using a calibrated 'A' grade 5ml pipette after 30 seconds and again after 5 minutes incubation at 37°C (Preliminary experiments had shown that all measurable cellulase activity was completed within 5 minutes). A commercial enzyme was also tested to show the relative activity of the *Trichoderma* culture filtrates. All assays were carried out in duplicate.

A 5 ml 'A'grade pipette was calibrated prior to the assay by measuring the flow rate of 5 ml CMC buffer; inactive filtrate; and CMC and distilled water.

Results were calculated as a mean % loss in viscosity after 5 minutes incubation at 37°C:

$$\frac{Vo-Vt}{Vo-Vb} \times 100$$

Where: Vo = flow time after 30 seconds.

Vt = flow time after 5 minutes incubation.

Vb = flow time of buffer.

2.3.4.3 Results

The results of the quantitative assay for cellulase activity by *Trichoderma* isolates can be seen in tables 2.8 a and b:

Trichoderma isolate	CMC + Glucose	CMC - Glucose
Trichoderma aureoviride SIWT.1	64.4	44.1
T. harzianum IMI 206040	40.0	38.2
T. polysporum IMI 206039	40.0	29.4
T. viride IMI 24039	35.6	41.2
T. viride IMI 49791	55.6	44.1
Trichoderma CCA sample	44.4	26.5
Trichoderma FYT strain	62.2	29.4
T. viride IMI 335517	31.1	41.2
T. harzianum IMI 335518	60.0	35.3
T. pseudokoningii S.I.W.T 22	62.2	32.4
T. harzianum S.I.W.T 25	68.9	29.4
T. pseudokoningii S.I.W.T 33	28.9	32.35
T. pseudokoningii S.I.W.T 51	53.3	41.2
T. pseudokoningii S.I.W.T 55	68.9	41.2
T. pseudokoningii S.I.W.T 64	62.2	26.5
Trichoderma S.I.W.T 140	42.2	38.2
Trichoderma (ASH)	64.4	41.2
T. citrinoviride IMI 335519	62.2	29.4
T. saturnisporum IMI14685	57.8	41.2
T. longibrachiatum IMI 536408	51.1	15.8
T. reesei IMI 192656ii	44.4	26.5
T. saturnisprorum S.I.W.T 142	40.0	20.6

Table 2.8.a Cellulase activity of *Trichoderma* isolates incubated at 25°C. Figures represent the mean % loss in viscosity of CMC solution after 5 minutes incubation at 37°C with filtrates from different *Trichoderma* isolates.

Trichoderma isolate	CMC + Glucose	CMC - Glucose
Trichoderma viride SIWT11	44.4	44.1
T. viride S.I.W.T 14	42.2	32.4
T. viride S.I.W.T 24	43.7	38.2
T. viride S.I.W.T 28	40.0	38.2
T. viride S.I.W.T 30	57.8	32.4
Trichoderma S.I.W.T 38	40.0	29.4
T. viride S.I.W.T 40	44.4	47.0
T. viride S.I.W.T 43	42.2	35.3
T. viride S.I.W.T 53	55.5	32.3
T. viride S.I.W.T 60	37.8	44.1
T. viride S.I.W.T 67	44.4	38.2
T. viride S.I.W.T 70	66.7	41.2
T. viride S.I.W.T 90	71.1	35.2
T. viride S.I.W.T 100	64.4	38.2
T. viride S.I.W.T 110	48.8	44.1
T. hamatum S.I.W.T 150	64.4	38.2
Trichoderma S.I.W.T 170	51.1	35.3
Trichoderma S.I.W.T 190	62.2	38.2
T. polysporum S.I.W.T 220	42.2	38.2
T. hamatum S.I.W.T 4	73.3	47.0
T. polysporum S.I.W.T 13	48.9	32.35
T. saturnisporum S.I.W.T 91	37.8	47.1
T. hamatum S.I.W.T 44	26.7	38.2
T. polysporum S.I.W.T 200	48.9	47.1
T. saturnisporum S.I.W.T 69	42.2	44.1

Table 2.8.b Cellulase activity of *Trichoderma* isolates incubated at 22°C. Figures represent the mean % loss in viscosity of CMC solution after 5 minutes incubation at 37°C with filtrates from different *Trichoderma* isolates.

Little difference in % loss of viscosity was recorded in the duplicate samples (see appendix 3).

In contrast to amylase and pectinase the presence of glucose in the growth medium has not had significant effects on the cellulase activity of most isolates. In most cases the activity is greatest in the presence of glucose however this is most likely due to enhanced growth of the organism rather than any inductive effect on enzyme production. Eight isolates show cellulase activities that are greater in the absence of glucose, namely *Trichoderma viride* IMI24039, *T. viride* IMI335518, *T. pseudokonigii* SIWT. 33, *T. viride* SIWT. 40, *T. viride* SIWT. 60, *T. polysporum* SIWT 220, *T. saturnisporum* SIWT. 91 and *T. hamatum* SIWT. 44. The differences in mean % drop in flow time between the two medium types for most of these isolates is small and are unlikely to indicate catabolite repression.

2.3.4.4 Discussion

The use of CMC to measure cellulase production is a convenient method as the compound is soluble in water (unlike native cellulose (Farmer, 1967)). Various authors have used CMC to measure the endo-glucanase activity (C_x) of different fungal species (Highley, 1973; Bisaria and Ghose, 1981; Sandhu and Kalra, 1982; Taj-Aldeen, 1993; Archer and Wood, 1994 and Cai *et al*, 1994). This activity was considered to be a precursor to cellulose breakdown by Eriksson *et al* (1974) as the endo-glucanases act randomly over the cellulose chain and other cellulase enzymes (exo-glucanases and glucosidases) breakdown the material further. (Bisaria and Ghose, 1981 and Priest, 1984).

The results of the assay (tables 2.8a and b) show that in most cases the production of cellulase was increased in medium containing glucose. These results are similar to those seen with brown rot fungi (Highley, 1973) and *Trichoderma longibrachiatum* (Sandhu and Kalra, 1982), where the presence of other carbon sources increased the production of cellulases to degrade CMC. This stimulation was thought to be linked to higher biomass production on the growth medium as the enzymes are constitutive.

The isolates that showed the largest cellulase activity were not those species normally associated with commercial cellulase production i.e. the *Trichoderma reesei* isolate used did not show a higher enzyme activity. This may be due to this isolate (*T. reesei*) not being ideally suited to the minimal growth medium used and hence not producing large quantities of the required enzymes or may be indicative that large interspecies variation exists in the production of cellulases. As with all *in-vitro* tests this method has the disadvantage that the results only indicate how the organisms performed in a particular trial under controlled conditions. How the chosen organisms will perform in wood under field conditions can only at present therefore be hypothesised.

2.4 DISCUSSION

From these experiments it is evident that the range of growth of *Trichoderma* isolates is varied. Morton and Eggins (1977) showed that growing different organisms (including *Trichoderma*) over a range of temperatures affected the growth rate of the tested organisms. This was also observed by Eastburn and Butler (1991) who investigated the effect of a range of temperatures (3-40^{o}C) on the growth of *Trichoderma harzianum*. If an isolate is to be used in the field it must be able to grow throughout a range of temperatures. Growth at various temperatures does not of course guarantee that the organism will produce degradative enzymes under these conditions or indeed how active any such enzymes, if produced, will be.

The nutrient composition of the medium will affect the growth of the isolates and also the secretion of enzymes. Using a minimal medium with a C:N ratio similar to that of wood it is expected the isolates that show the best enzyme activity on such a medium are likely to give the best increases in permeability when exposed to the timber samples under field conditions. On the basis of this assertion and with regard to the temperature growth ranges of the *Trichoderma* isolates, 5 were selected in the first instance to be tested against small wood

block samples. A further isolate (*T. viride* SIWT 100) was added at a later date as a negative control to show the effects on wood permeability of an isolate with poor enzyme activity. The isolates selected for further work were:

1) *Trichoderma aureoviride* SIWT.1
2) *Trichoderma polysporum* IMI 206039
3) *Trichoderma pseudokoningii* S.I.W.T. isolate 51
4) *Trichoderma pseudokoningii* S.I.W.T isolate 64
5) *Trichoderma viride* S.I.W.T. isolate 70
6) *Trichoderma viride* S.I.W.T. isolate 100

Organisms 1 and 5 were selected because of the larger cellulase action observed in the quantitative assay and because they showed significant amounts of pectinase activity. Organisms 2 and 4 were selected on the basis of showing the highest pectinase activity. Organism 3 was selected on the basis of high amylase activity and significant pectinase and cellulase activity. Organism 6 was included as a negative control as it showed no pectinase activity and relatively low cellulase activity in the assays.

Sugars influence the induction/ repression of the selected enzymes. If these organisms are to able to improve the permeability of wood which will contain carbon sources then the selected organisms should be capable of producing the enzymes under sugar rich conditions.

2.5 CONCLUSIONS.

All the selected *Trichoderma* isolates used in this work will grow over a wide range of temperatures although growth rate may be reduced to relatively low levels as lower temperatures are reached.

Isolates also showed abilities to produce significant amounts of the various degradative enzymes under nutrient conditions similar to those expected to be found in wood.

Most *Trichoderma* isolates tested exhibited catabolite repression in pectinase and amylase production but not with cellulase production.

Chapter 3. Permeability Determinations using Decalin.

3.1 INTRODUCTION

Wood permeability is dependent on the different structural properties of the tested wood samples. Different methods exist to evaluate wood permeability and hence the choice of a suitable method was of primary importance in the development of this project. Two types of method exist and both have been used at different stages of this project; a flow method (air permeability, Chapter 5) and a fluid uptake method using a suitable liquid (section 3.2). Fluid uptake has the advantage of being rapid, allowing a high degree of replication and permits relatively simple calculations of permeability changes.

Petroleum spirit and similar fluids have been used to evaluate the permeability of wood samples (Petty,1975; Pendlebury *et al*, 1991). Decahydronaphthalene (decalin) is recommended in British Standard BS5707 (1979) for the testing the penetration of preservative and has been used to evaluate permeability (Carey,1980; Schoeman 1994). This liquid was selected to measure the permeability of wood samples in this project as previous researchers had been able to detect increases in permeability of window frame joints after exposure to various micro-organisms (Carey 1980).

The growth of microorganisms through green wood is generally thought to be difficult because of the high moisture contents found in the timber and the fact that the plant defences are still physiologically active. A preliminary study into the ability of *Trichoderma* isolates to colonise freshly felled timber was undertaken. This would demonstrate whether the organisms were capable of growing through the timber. Using a fluid uptake method for determining changes in permeability, an assessment of whether the isolates were capable of improving the permeability of the timber would be possible.

3.2 LIQUID UPTAKE METHOD FOR DETERMINING PERMEABILITY

3.2.1. Introduction.

Decalin is a simple hydrocarbon liquid that has been shown to readily penetrate into wood and has been used to test the penetration of preservative into wood samples (under pressure). This method was used by Carey (1980) to determine if the permeability of window joints were improved after exposure to various organisms and whether paint would be more readily absorbed into these joints. The permeability of the wood sample is related to the uptake of decalin with time, hence more permeable samples will absorb more liquid over a set time period. The aim of the following experiments was to demonstrate the suitability of a liquid uptake method to show changes in wood permeability caused by *Trichoderma* species.

3.2.2 Method.

This method relies on wood blocks being dry, cut from the same direction in the tree and submerged in the same orientation in the liquid. Initially 50 x 25 x 15 mm blocks of Scots pine (*Pinus sylvestris*) sapwood were cut from air dried wood and the blocks were then dried at 105^{o}C overnight before treatment with the solvent. Blocks were stored over desiccant prior to weighing. The dry weight of the blocks was measured and the blocks were submerged in Decahydronaphalene (decalin) with the large longitudinal tangential face uppermost for 10 seconds. The blocks were then blotted dry on paper towel until no solvent mark was left on the towel by the blocks, treated blocks were then reweighed and the treatment was repeated for a further 10 seconds, reblotted and reweighed to give an uptake weight (g) after total immersion time of 20 seconds. This treatment was repeated to give results for the following time periods 10, 20, 30, 40, 50, 60, 90, 120, and 180 seconds.

In total the treatment was repeated on 10 replicates and the % increase in weight of each block was calculated :

$$\frac{\text{Wet weight of block - Dry Weight of block}}{\text{Dry Weight of block}} \times \frac{100}{1}$$

3.2.3 Results

The results for decalin uptake into dried Scots pine blocks can be seen in figure 3.1.

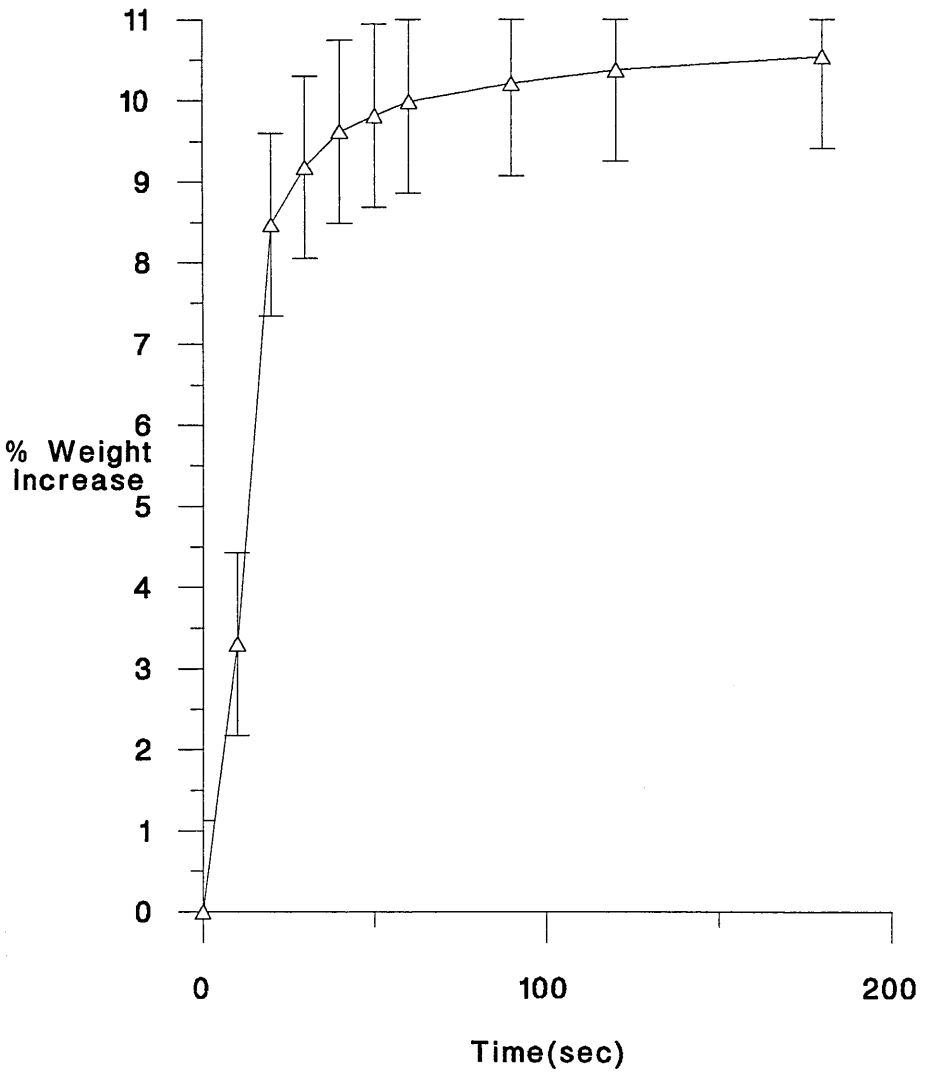


Figure 3.1 Mean % uptake of decalin (for 10 replicates) into Scots pine sapwood blocks over 180 seconds. N.B. Error bars were calculated as the standard error of the data set.

The results in figure 3.1 show that uptake of decalin is initially rapid, however the rate of uptake slows over the duration of the dipping.

3.2.4 Discussion.

From figure 3.1. it can be seen that initially there is a rapid uptake of decalin over the first 60 seconds after which the uptake rate slows significantly. Over the time period sampled little further uptake occurs after the initial 60 seconds. Beyond the sampled time period the uptake will continue to rise until saturation of the blocks is achieved although it will be at a considerably reduced rate. With more permeable wood samples a faster rate of fluid uptake would be expected and hence it may be possible to differentiate wood types since rates will differ from species to species. If the blocks were dipped until saturation, differences may not be observed as the samples may show similar total uptakes but at different rates. Hence for permeability determinations it may be preferable to submerge the blocks for only 10 seconds as uptake will still be exponential at this point and should differentiate between woods of different permeabilities more effectively.

3.3 PERMEABILITY OF DIFFERENT WOOD SPECIES.

3.3.1 Introduction

The permeability of wood species is expected to be different because of the variation that exist in the composition of wood i.e. differences in shapes, sizes and condition of bordered pits and tracheids. The aim of the experiments in this section was to investigate if differences in decalin uptake were observed between two timber species (Scots pine and Sitka spruce (*Picea sitchensis*)) and whether differences could be measured between the heartwood and sapwood of the two species.

3.3.2 Methods

Blocks (15mm cubes) were cut from air dried Scots pine and Sitka spruce sapwood and heartwood. These were cut to ensure that the permeability could be measured in the longitudinal, radial and tangential direction. Blocks were oven dried overnight at 105^{o}C prior to being weighed and then dipped in decalin for 10, 20, 30 and 60 seconds (as in 3.2.2). Fifteen replicates were used in this experiment and the resulting % uptake values were calculated as in section 3.2.2.

3.3.3 Results

The results of the uptake of decalin into Scots pine and Sitka spruce samples can be seen in figure 3.2.

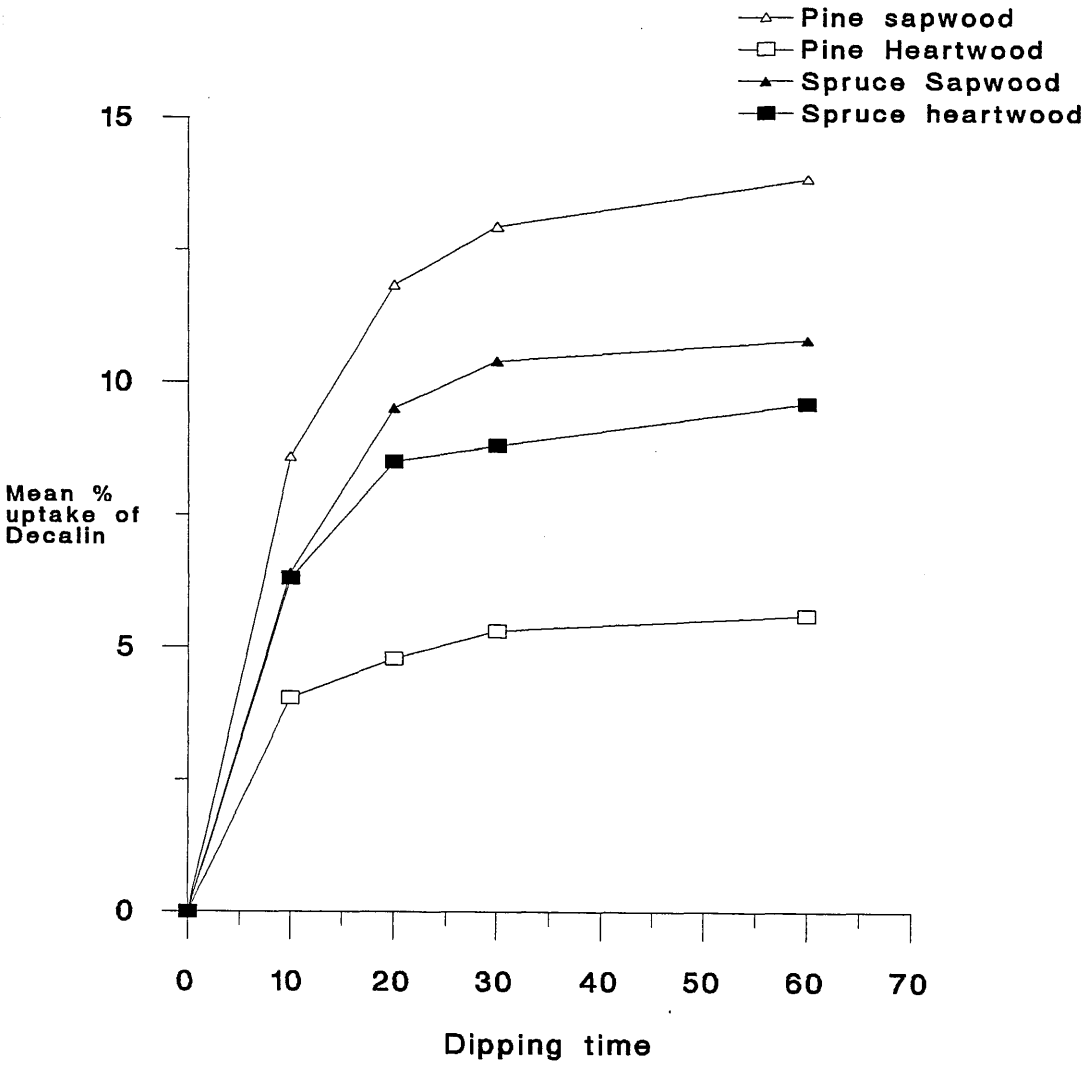


Figure 3.2 Mean % uptake of decalin in Scots pine and Sitka spruce sapwood and heartwood over 60 seconds (results are a mean of 15 replicates). N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The results in figure 3.2 show the different uptakes of decalin over a 60 second period. In both species the sapwood material showed a higher uptake in decalin than the corresponding heartwood of same species. The pine sapwood showed the highest mean % uptake (14%)

with pine heartwood showing the lowest uptake (6.5%) over the 60 seconds immersion period.

3.3.4 Discussion.

From figure 3.2 it is possible to see that there is a difference in average uptake of decalin over the 60 second period. Differences in uptake will occur because of different diameters of the cut fibres and differences in the size/ shape of pit membranes which will alter the rate and limit the total uptake of the fluid. Drying the wood samples under different regimes have been shown to alter the permeability of samples (Erikson and Crawford, 1959; Comstock and Cote, 1968), at 105^{o}C most of the interconnecting pits are likely to be aspirated thereby reducing the uptakes. Preventing pit aspiration has been shown to increase the permeability of the wood and has been achieved by many different methods e.g. solvent exchange drying (Thomas and Nicholas, 1966; Petty, 1978; Cutter and Phelps, 1986), steaming (Nicholas and Thomas, 1968 (a); Cutter and Phelps, 1986), impregnation of degradative enzymes (Nicholas and Thomas, 1968(b); Bauch *et al*, 1970; Imanura *et al*, 1974; Morishita *et al*, 1986; Militz, 1993 a & b) and ponding (biological degradation) (Dunleavy and Fogarty, 1971; Unligil, 1972; Bergman, 1986; Bjurman and Holappa, 1989).

3.4 EFFECT OF AUTOCLAVING ON DECALIN UPTAKE.

3.4.1. Introduction

Since steaming has been shown to improve the permeability of refractory timber to preservative (Nicholas and Thomas, 1968 (a)), and autoclaving is a method of steam sterilisation it would be highly likely that autoclaving may also alter the permeability of wood blocks. Since a method was required to sterilise dried wood samples prior to exposure to *Trichoderma* isolates the aim of the following experiment was to investigate the effect of the autoclaving process on the uptake of decalin in different wood samples.

3.4.2 Methods

Eighteen blocks of Scots pine sapwood were cut to 50 x 25 x 15 mm (as in 3.2.2); nine blocks were wrapped in aluminium foil and autoclaved for 15 minutes at 121^{o}C while the remainder were left as controls. After autoclaving the treated and control blocks were dried at 105^{o}C to constant weight. The blocks were then dipped using the method described in section 3.2.2. for 10, 20, 30, 40, 50, 60, 90, 120 and 180 seconds. This extended dipping period was examined to determine whether autoclaving would open up the wood structure thereby prolonging the period of rapid decalin uptake. The results were analysed by analysis of variance to establish if the changes in decalin uptake were significant.

3.4.3. Results

The results of the uptake of decalin into control and autoclaved Scots pine sapwood can be seen in figure 3.3.

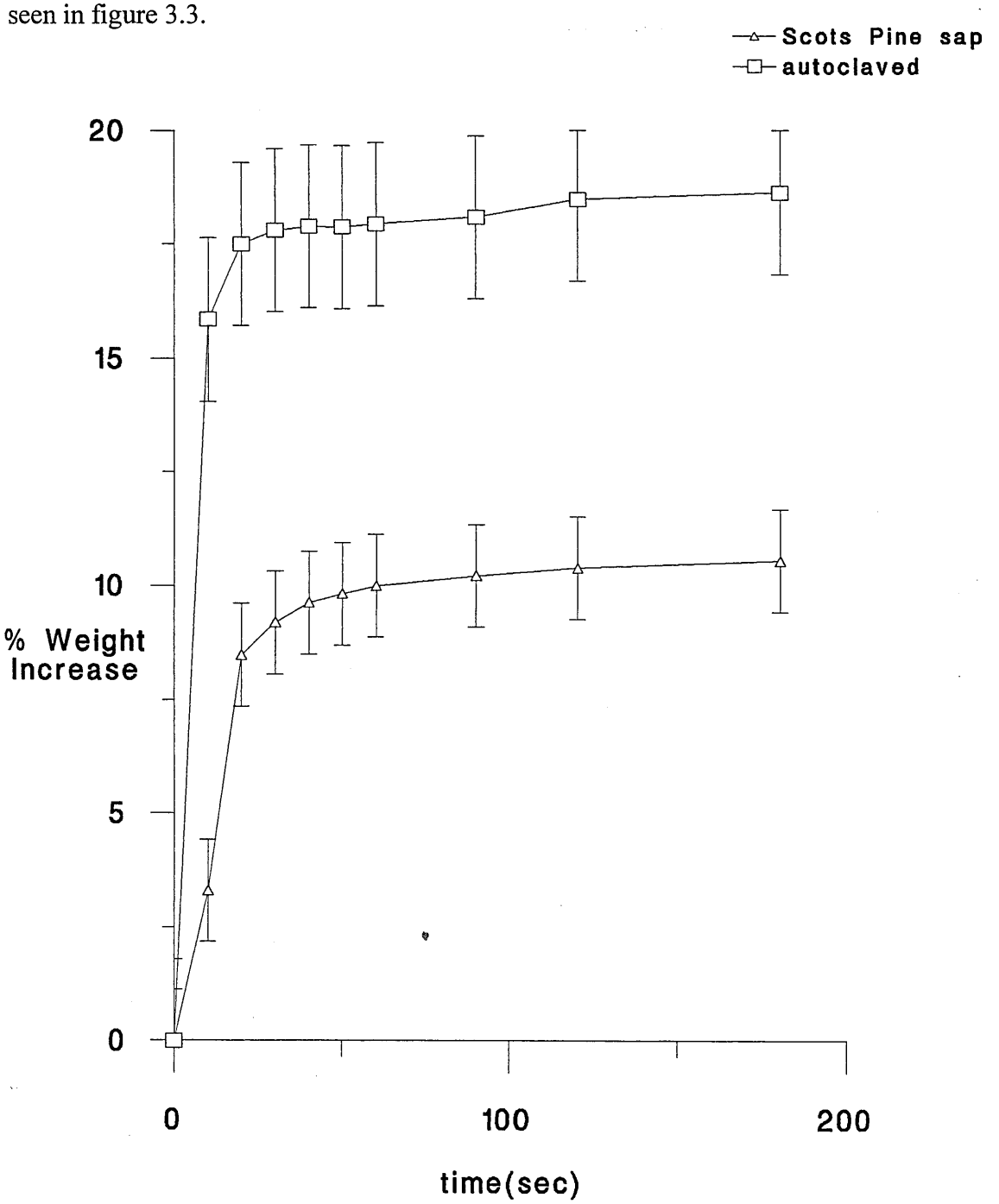


Figure 3.3 Mean % uptake of decalin in Scots pine sapwood with and without autoclaving (results for each treatment are a mean of 9 replicates). N.B Error bars were calculated as the standard error of the data set.

The results in figure 3.3 show that autoclaving increases the uptake of decalin especially over the initial 10-20 seconds. Statistical analysis of the effect of autoclaving on the uptake of decalin into Scots pine sapwood can be seen in table 3.1:

Wood Sample	No.	Mean % uptake after 180 seconds	F-value	P- value
Control sapwood	9	10.4(0.6)	22.87	0.000
Autoclaved sapwood	9	18.5(1.9)		

Table 3.1 Results of Anova. Showing differences between the uptake of decalin into control and autoclaved Scots pine sapwood, the F-value indicates that the permeabilities are significantly different at 95% confidence intervals.

While it is obvious that the permeabilities of the two populations are different, increased uptake of decalin occurs within the first 10-20 seconds of dipping after which rates of uptake appear similar.

3.4.4 Discussion.

Erickson and Crawford (1959) found that steaming green wood made no significant difference to the permeability as water passed readily through the sapwood of Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) and Western Hemlock (*Tsuga heterophyla* (Raf.) Sarg.), and steaming does not create new openings or alter the existing capillaries before the wood is dried. However when these same samples were dried the permeabilities were seen to be higher than in unsteamed wood seasoned in the same way, hence steaming the wood prior to seasoning prevents aspiration of some of the pit tori resulting in a higher permeability after drying.

Autoclaving wood blocks does significantly increase the permeability of the wood as the amount of decalin flowing into the wood increases. The work of Nicholas and Thomas (1968a) showed that pit membranes were disrupted by steam, and pressure deaspirated the membranes. These effects explain why the uptake of decalin was increased in the steamed wood. The shape of the graphs produced for both autoclaved and control samples (figure 3.3) are very similar with rapid uptakes of decalin occurring within the first 10-20 seconds. This may indicate that while the exterior regions of the block are becoming saturated with the fluid, air trapped in the interior regions of the blocks is greatly slowing the continuing rate of fluid uptake. Autoclaving would appear to have had little effect on the ingress of the decalin to such inner regions but has significantly increased the rapid early uptake of the fluid.

3.5. EFFECT OF AUTOCLAVING ON DECALIN UPTAKE BY DIFFERENT SIZES OF WOOD BLOCK.

3.5.1 Introduction.

The size of wood blocks is important in sterilisation as larger blocks require longer time periods for effective sterilisation to allow the steam to completely penetrate samples. The aim of the following experiments was to investigate the effect of wood block size (after autoclaving) on the uptake of decalin in both Scots pine sapwood and heartwood blocks.

3.5.2. Methods.

Blocks of Scots pine sapwood and heartwood were cut to two different sizes 50 x 25 x 15mm and 30 x 15 x 10mm (18 replicates of each size). Half of the cut blocks of each size were autoclaved (as in section 3.4.2) and dried down at 105^{o}C to constant weight. The remaining untreated control blocks were simply dried down at 105^{o}C to constant weight as before

(section 3.2.2.). The blocks were then weighed and dipped in decalin for a total of 10 seconds and then blotted dry prior to weighing.

3.5.3 Results

The effect of autoclaving on the mean % uptake of decalin in large sized blocks (50 x 25 x 15mm) is shown in table 3.2:

Wood Type	No.	Mean % uptake	St. Deviation	F-value	P-value
Control sapwood	9	6.116	2.057	22.07	0.000
Autoclaved sapwood	9	11.758	2.872		
Control heartwood	9	1.957	0.667	6.46	0.022
Autoclaved heartwood	9	2.759	0.671		

Table 3.2 Mean % uptake of decalin for large sized wood blocks.

These results again show that there is an increase in decalin uptake in both pine sapwood and heartwood after steaming. The analysis of variance shows that the increase in permeability after autoclaving in both sapwood and heartwood are significant for 95% confidence intervals for the means.

The results of the effect of autoclaving on the uptake of decalin in small sized (30 x 15 x 10mm) wood blocks can be seen in table 3.3

Wood Type	No.	Mean % uptake	St. Deviation	F-value	P-value
Control sapwood	9	13.920	4.819	11.10	0.004
Autoclaved sapwood	9	20.286	3.261		
Control heartwood	9	7.467	0.710	56.75	0.000
Autoclaved heartwood	9	13.166	2.279		

Table 3.3 Mean % uptake of decalin for small sized wood blocks.

The results illustrated in table 3.3 indicate increases in permeability in both sapwood and heartwood when wood blocks are autoclaved are much larger than in the large sized wood blocks (table 3.2). The analysis of variance of the fluid uptake into both the small sapwood and heartwood blocks again shows that the increase after autoclaving is significant for the 95% confidence interval for the means. Smaller blocks have a greater mean % uptake of decalin compared with the larger blocks which is probably caused by the higher surface area : volume ratio in the smaller blocks.

3.5.4 Discussion

Nicholas and Thomas (1968(a)) showed that the permeability of Loblolly pine (*Pinus taeda*) was increased when steamed. Steaming disrupted the pit membranes preventing aspiration and hydrolysed some of the carbohydrate components (Excoffier *et al*, 1991) of the membranes and hence gave rise to a higher permeability when dried. In this study all autoclaved wood blocks showed the increase in uptake of decalin in the wood, but the extent by which the decalin uptake increased was dependent on the size of the blocks. Larger blocks have a lower surface area to volume ratio and hence in 10 seconds dipping less decalin (% by weight increase) could be absorbed into the block.

Smaller blocks showed significant increases in decalin uptake and the magnitudes of these increases were again bigger than in the large blocks. The effects of steaming may have increased the permeability of the smaller blocks by a greater proportion as the steam would penetrate further into the blocks and disrupt more of the available pit membranes. Steaming could dissolve those compounds (e.g. sap sugars and hemicellulose) in the wood blocks that affect the permeability of the blocks and hence after drying the permeability is seen to be higher.

Heartwood by definition is found in the heart of the tree and performs only a few functions in the standing tree. This material is transformed sapwood in which the transport mechanism is disrupted i.e. pits are aspirated and encrusted with extractives limiting the permeability of the wood (Liese and Bauch, 1967; Siau, 1984). The main functions of the heartwood are support and to lesser extent storage of extraneous materials (Hillis, 1987). With the initial permeability of the heartwood being lower and the pit membranes being encrusted with extraneous material it was possible that autoclaving would have a smaller effect on this permeability than in the sapwood region. Despite the autoclaving process increasing the permeability of the heartwood samples the final percentage uptake values have remained below that of the corresponding sapwood samples especially in the large blocks. Steaming is used for the sterilisation of wood blocks for various standard tests e.g. BS6009 (1987), however the time periods suggested are longer than those used in this experiment and are therefore likely to further influence the permeabilities of the wood. The increases in permeability recorded here and changes in soluble nutrients associated with autoclaving making sterilisation by this method unsuitable for use in experiments where the effects of colonising organisms on the wood permeability are to be measured.

3.6 DECALIN UPTAKE VIA EXPOSED FACES OF WOOD BLOCK SAMPLES.

3.6.1 Introduction

Wood component structures run in different directions within the standing tree i.e. tracheids run longitudinally up and down the tree and rays run in a radial direction. These components contribute independently to the permeability of the timber in both softwoods and hardwoods, and hence permeability varies depending in which direction it is measured. Generally the permeability will be greatest in the longitudinal direction, with the permeability in the radial direction being lower and the tangential permeability being the lowest (Comstock, 1970). Longitudinal permeability is generally highest as tracheids run in this direction and in the

living tree sap is transported in this direction and hence the material is very permeable in the green state (Banks, 1970). Radial permeability is lower as only the ray material truly runs in this direction. Permeability in this direction is dependent on the proportions of ray material and tracheid fibres. Tangential permeability will be lower still as this permeability is reliant on flow only through tracheid fibres.

The aim of the following experiment was to investigate if directional differences in permeability could be detected using the decalin uptake method.

3.6.2 Methods.

Samples of Scots pine and Sitka spruce sapwood and heartwood were cut into 15mm cubes (to ensure that each face had the same surface area). The cubes were then dried at 105^{o}C and 4 sides of each cube were sealed with paraffin wax to leave open two opposite sides exposing faces with the same orientation to allow measurement of uptake in solely the longitudinal, radial or tangential directions. This was achieved by covering the two faces to be left exposed with tape before immersing the block into molten paraffin wax. After the wax had solidified the tape was removed to expose the required cube faces. Control blocks were left with all faces exposed. Ten replicates were prepared to measure the flow in each of the corresponding directions and controls. The cubes were then weighed and submerged in decalin (section 3.2.2) before being carefully blotted dry (to avoid removing any of the wax from the sealed surfaces). The cubes were dipped for 10, 20, 30, and 60 seconds and could be compared with the earlier cubes used in section 3.2.2.

3.6.3 Results.

The results of the uptake of decalin in different flow directions in Scots pine sapwood and heartwood blocks are shown in figure 3.4. The results indicate that Scots pine sapwood is more permeable than the heartwood in all directions and that uptake is dependant on the flow direction. The uptake in the individual directions show differences as expected with flow in the longitudinal direction greatest, followed by flow in the radial direction, with flow in the tangential direction being the lowest.

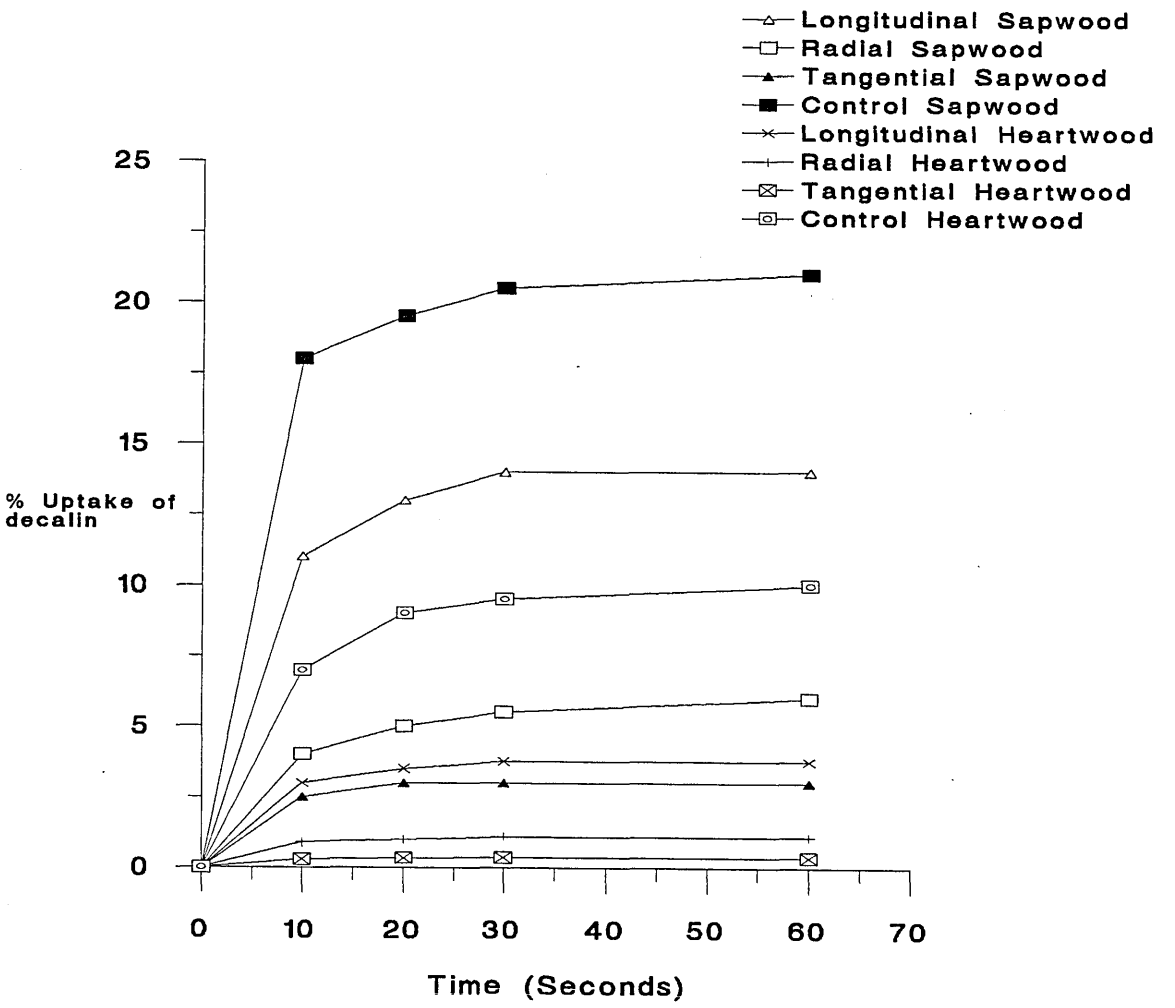


Figure 3.4 Mean % uptake of decalin by Scots pine sapwood and heartwood in control blocks and longitudinal, radial and tangential flow directions for 10 replicate blocks. N.B.

Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The results for the uptake of decalin in different flow directions in Sitka spruce sapwood and heartwood can be seen in figure 3.5:

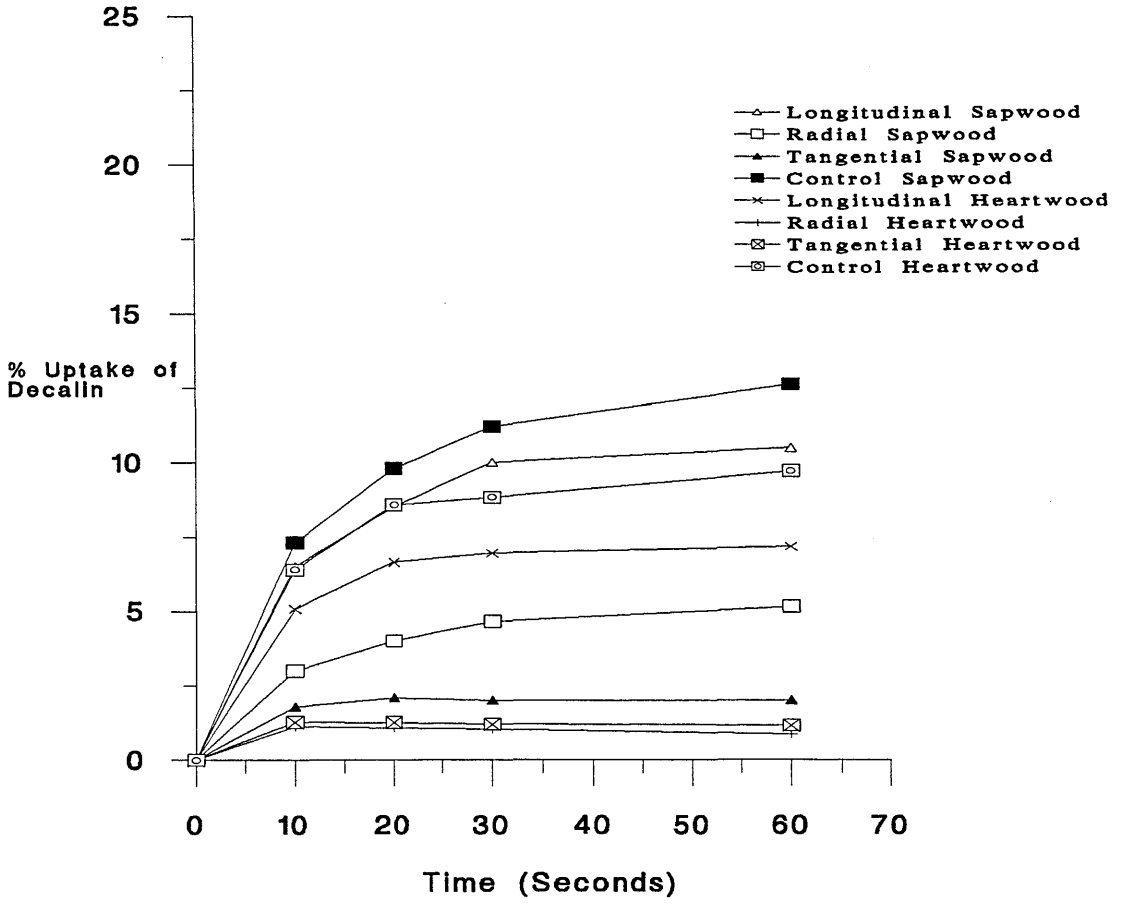


Figure 3.5 Mean % uptake of decalin by Sitka spruce sapwood and heartwood in control blocks and longitudinal, radial and tangential flow directions for 10 replicate blocks. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The results (figure 3.5) show that although spruce sapwood is more permeable than spruce heartwood both have a much lower uptake than that of Scots pine sapwood and uptake via the longitudinal direction is much greater than that in either the radial or tangential directions. In

the heartwood uptakes for the radial and tangential directions are similar, however in the sapwood radial uptake is greater than in the tangential direction.

3.6.4 Discussion.

The differences in permeabilities between the wood types are illustrated in figures 3.4 and 3.5. Comstock (1968) showed that the permeability of Scots pine wood samples are several times greater than similar Sitka spruce material. As expected the permeability of the sapwood material is higher than in heartwood for both species (Siau 1984). This is due to the encrustation of pits during differentiation of the heartwood which reduces the permeability.

The differences in directional permeability are due to different structures in the wood influencing the permeability, figure 3.6 illustrates the structural differences between the different flow directions.

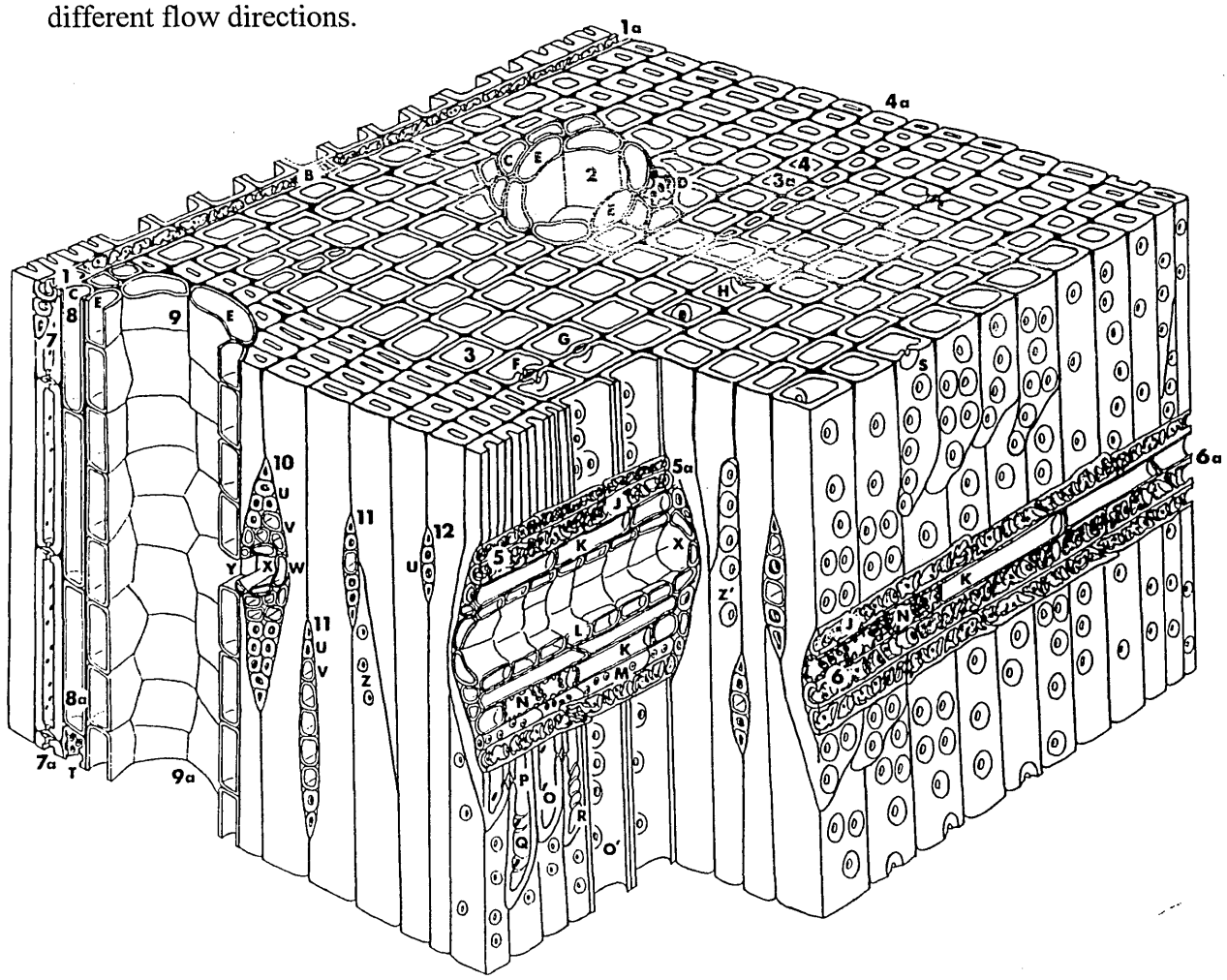


Figure 3.6 Gross structure of a typical southern pine softwood (from Siau, 1984). Transverse view, 1-1a ray; B dentate ray tracheid; 2 resin canal; C thin-walled longitudinal parenchyma; E epithelial cells; 3-3a earlywood tracheids; F radial bordered pit pair cut through torus and pit apertures; G pit pair cut below pit apertures; H tangential pit pair; 4-4a latewood. Radial view 5-5a sectioned fusiform ray: J dentate ray tracheid; K thin walled parenchyma; L epithelial cells; M unsectioned ray tracheid; N thick-walled parenchyma; O latewood radial pit (inner aperture); O' earlywood radial pit (inner aperture); P tangential bordered pit; Q callitroid like thickenings; R spiral thickening; S radial bordered pits (the middle lamella has been stripped away, removing crassulae and tori); 6-6a sectioned uniseriate heterogeneous ray.
Tangential view, 7-7a strand tracheids; 8-8a longitudinal parenchyma (thin-walled);T thick-walled parenchyma; 9-9a longitudinal resin canal; 10 fusiform ray; U ray tracheids; V ray parenchyma; W horizontal epithelial cells; X horizontal resin canal; Y opening between

horizontal and vertical resin canals; 11 uniseriate heterogeneous rays; 12 uniseriate homogeneous ray; Z small tangential pit in latewood; Z' large tangential pits in earlywood.

From this diagram it is possible to visualise how the permeabilities in the different directions are related. In the longitudinal direction the tracheids and resin canals will carry fluid. These structures are longer and the number of pit openings to be crossed within a set distance will be fewer than in either the radial or tangential directions. Since it is the condition and number of pit openings that have to be crossed that limits the permeability, hence in this direction the permeability in the longitudinal direction is higher (Comstock, 1970). Ray material runs in the radial direction transporting water and nutrients radially within the tree. Tracheids may also be required to be traversed in this direction as longitudinal fibres have crossfield pits in their cell walls and at the interconnecting ends of the tracheids, which will also influence flow of fluids radially through the wood samples (Siau, 1984). The permeability in this direction will be lower than in the longitudinal direction as the number of tracheids to be crossed and the number of pit membranes to be negotiated over a given distance will be higher than in the longitudinal direction. Permeability in the tangential direction will be lower than in the radial direction as the flow in this direction is dependant solely on bordered pits in tracheids and not ray material. McQuire (1970) suggested that it was the radial permeability of wood sections that has the largest implication for timber preservation as this flow direction is taken by preservative into roundwood logs during treatment. Therefore any biological pretreatment process expected to improve preservative treatment of roundwood material should be expected to significantly increase permeability in the radial direction.

3.7 EFFECTS OF DIFFERENT DRYING REGIMES ON DECALIN UPTAKE.

3.7.1 Introduction.

Different drying conditions have been shown not to alter the strength properties of Scots pine wood samples (Comben, 1955), but to alter the state of pit membranes in the wood through aspiration. On drying wood the air permeability can be reduced to between 1 and 5% of that for green wood depending on the intensity of heat used (Siau, 1984). Since drying of the blocks is an essential stage in measuring permeability by both decalin uptake and air permeability methods it is essential to establish whether different drying regimes will themselves effect wood permeability. These experiments therefore aim to investigate the effect of drying fresh and frozen wood blocks by different regimes/ temperatures on subsequent decalin uptake measurements.

3.7.2 Methods

Four different drying regimes were used to assess the effect of drying fresh Sitka spruce on subsequent permeability measurements.

1) Ambient temperature over desiccant.

2) 50^{o}C in an oven.

3) 50^{o}C over desiccant in an oven.

4) 103^{o}C in an oven.

Fifteen mm cubes of Sitka spruce sapwood were dried following each of the above regimes to a constant weight. Eighteen replicate blocks were used for each regime. The above experiment was repeated using cubes frozen at -20^{o}C for 24 hours prior to drying to establish if freezing the wood had any effect on drying or subsequent decalin uptake. Five cubes from each treatment were dried down at 105^{o}C in an oven to constant weight on completion of

each drying regime to determine the amount of residual moisture left in the blocks. The % uptake after10 seconds immersion in decalin was then measured on the remaining 13 replicates from each treatment group (section 3.2.2).

3.7.3 Results

The results in table 3.4 show the mean % uptake of decalin by fresh and frozen Sitka spruce sapwood after drying to constant weight under different regimes.

Drying Regime	No. Blocks	% Mean uptake	St. Dev	Residual moisture content (%)
Fresh Air dried (O.D.)	13	28.44	6.23	4.65
Fresh 50°C	13	28.44	7.44	0.94
Fresh 50°C (O.D)	13	29.62	3.68	3.74
Fresh 103°C	13	28.36	4.65	0.38
Frozen Air dried (O.D)	13	26.42	6.42	4.82
Frozen 50°C	13	29.79	6.35	2.13
Frozen 50°C (O.D)	13	28.32	4.75	4.02
Frozen 103°C	13	25.37	8.53	0.88

Table 3.4 Mean % uptake of decalin after fresh or frozen Sitka spruce has been dried to constant weight using different drying regimes (13 replicates for each treatment group). N.B. "O.D" represents drying over desiccant. Residual moisture content shows the moisture remaining in the blocks after each temperature/ drying regime (5 replicates).

The results in table 3.4 show the mean % uptake on decalin after the blocks have been dried down to constant weight. When the uptakes were analysed statistically no significant differences in decalin uptake were observed with the different drying regimes (table 3.4). It is

notable that blocks dried over desiccant had slightly higher residual moisture contents however this did not affect subsequent decalin uptake.

3.7.4 Discussion.

Drying of wood under different conditions has been reported (Siau, 1984; Booker, 1990) to have an effect on the subsequent permeability of the dried wood. Solvent exchange drying has been shown to leave the permeability of timber higher after drying as the solvent prevents pit membranes from aspirating (Petty, 1978; Cutter and Phelps, 1986). Since hydrogen bonding is associated with aspiration, Thomas and Kringstad (1971) showed that when the water in the tracheids is replaced with solvents less bonding is seen between the pit tori and bordered pit cell walls and hence less aspiration occurs. The intensity of the heat used to dry the timber is also thought to contribute to the degree of aspiration (Siau, 1984). Using the decalin uptake method no significant differences were observed between the different drying regimes examined in this study. It may be possible that the decalin uptake method was not sensitive enough to differentiate between the drying regimes or simply that the drying regimes were not harsh enough to cause permeability differences. Using spruce which has a naturally low permeability may also have masked any measurable difference and changes in permeability of the dried timber would be incorporated within any natural variation in the timber.

From the results in table 3.4 it is possible to see that freezing has little measurable effect on the permeability of spruce sapwood and when analysed statistically showed no significant differences in permeability. Storage of blocks in a freezer to prevent mould infection prior to use is therefore feasible without adverse effect on wood permeability.

The results presented here which show that of a range of temperature/ drying conditions which could be used these conditions showed no significant differences in the permeability. Since drying at 103^{o}C is likely to result in complete aspiration and therefore lowest

permeability in the timber such wood will provide a good base level from which subsequent permeability increases caused by biological agents could be measured.

3.8 PRELIMINARY STUDY OF ROUNDWOOD COLONISATION BY *TRICHODERMA* SPP.

3.8.1 Introduction.

Green wood has been thought of as a fairly unfavourable environment for microorganisms to inhabit. The high moisture content and plant defence mechanisms may inhibit the growth of invading organisms. If an organism is to grow through green wood it must overcome these factors. The *Fungi imperfecti* (to which *Trichoderma* spp. belong) have been reported to act as primary colonisers of wood cut in the forest (Kaarik, 1974) and on occasion *Trichoderma* isolates have been isolated from standing trees (Koch and Thongjiem, 1989). The disabling of plant defences may allow the organism to grow through the plant more easily, even if the moisture content is at or near saturation point. The aim of this investigation was to determine if freshly felled Sitka spruce could be successfully colonised by *Trichoderma* and whether the disruption of the timbers natural defences could enhance the colonisation of the timber by the organism.

3.8.2. Methods.

Freshly cut Sitka spruce logs were randomly divided into two groups, one group was stored at +4^{o}C the other at -20^{o}C. The logs were approximately 12 cm in diameter and 30cm long. Ten logs were selected for the study, 6 were from the frozen logs and 4 from the refrigerated logs and were treated as follows:

i) Two frozen logs debarked (controls).

ii) Two frozen logs debarked and inoculated by painting a spore suspension (10%w/v) of *Trichoderma* from Binab FYT powder (AB Binab, Sigtuna Sweden) composed of

Trichoderma harzianum IMI 206040 and *Trichoderma polysporum* IMI 206039 spores) onto the surface of the logs.

iii) Two Frozen logs asceptically cored at a central location with a Matsun tree increment corer and Binab FYT pellets (composed of *Trichoderma harzianum* and *Trichoderma polysporum* spores)were placed in the bore hole and plugged with a portion of the core removed prior to inoculation.

iv) Two fresh logs debarked and inoculated as for ii) above.

v) Two fresh logs asceptically cored and inoculated as for iii) above.

After *Trichoderma* inoculation all logs were placed in autoclave bags which had a small hole left in the top to allow gas exchange. The logs were then incubated at ambient temperature in an unheated room for 9 or 14 weeks. After incubation the logs were cut in half. Three discs (3cm thick) were cut at equal distances along one half for microbial analysis. A further disc was cut from the central portion of remaining log and ten, 15mm cubes cut from the sapwood for permeability and moisture content determinations.

The three discs from one half of the log were sampled for microbial colonisers after being surface sterilised by passing the discs through a Bunsen burner. On the surface of the discs, two zones were identified according to the numbers of annual rings present i.e. zone 1 consisted of the first two annual growth rings in the sapwood and zone 2 was the identifiable heartwood region. Five chips were removed from the surface of each disc and plated out onto 3% Malt Extract agar and subsequent growth from all chips after 3 days incubation at 25^{o}C noted. The cubes cut from the logs were dried at 105^{o}C to constant weight and treated as in section 3.2.2.

3.8.3. Results

The results of plating out wood chips from different zones of sampled wood discs can be seen in table 3.5

		9 weeks		14weeks	
Treatment	**Disc**	**Sapwood**	**Heartwood**	**Sapwood**	**Heartwood**
Frozen uninoculated	1	B	B	B	
	2	B+T		B+T	B+T
	3	B+T		B+T	
Frozen pellet	1	B+T	B+T	B+T	B+T
	2	B+T	B+T	B+T	T
	3	B+T	B+T	B+T	B
Frozen spore	1	B+T	B+T	B+T	
	2	B+T	B+T	B+T	B+T
	3			B+T	B+T
Fresh pellet	1	B	B	B	
	2	B		B	
	3			B	B
Fresh spore	1	B	B	B	
	2	B+T	B+T	B	
	3	B+T	B+T	B	

Table 3.5 Isolations of organisms from wood chips removed from sapwood and heartwood regions of different log treatments. N.B. "B" represents growth of bacteria, "T" represents growth of *Trichoderma* from wood chips.

Trichoderma was isolated from the majority of inoculated frozen samples and less regularly from fresh samples, some *Trichoderma* was isolated from the uninoculated frozen samples, indicating that there was some contamination of the control logs.

The results of the moisture determinations and permeability measurements can be seen in table 3.6.

Treatment	9 weeks		14weeks	
	% Moisture Content (Dry Wt basis)	% Uptake of Decalin (Permeability)	% Moisture content (Dry Wt basis)	% Uptake of Decalin (Permeability)
Fresh uninoculated	190	17	182	28
Frozen uninoculated	200	20	211	59
Frozen pellet	151	16	190	56
Frozen spore	186	25	200	56
Fresh pellet	183	18	193	30
Fresh spore	199	20	202	28

Table 3.6 Moisture content and permeability of blocks removed from logs treated with *Trichoderma*. N.B. Blocks cut from fresh uninoculated Sitka spruce logs were included as extra controls

Results in table 3.6 show that there is little difference between the moisture contents of the log samples between sampling times. Higher permeabilities are seen in the frozen samples after 14 weeks.

The statistical analysis of moisture contents after 14 weeks shows no significant differences between treatments. This will be due to the logs being bagged and hence keeping the moisture content of the logs high. The permeabilities however showed differences between the treatments. Compared to fresh uninoculated control material; after 9 weeks only one treatment showed a significant increase at 95% confidence intervals this was the frozen spore. After 14 weeks 3 treatments showed a significant increase these were those samples that had previously been frozen.

3.8.4. Discussion

The isolation of *Trichoderma* from the different areas of the wood indicated that the organism was able to grow from the inoculum and colonise the wood despite the high moisture contents observed. Kaarik(1974) showed that most wood colonising organisms cannot colonise healthy green timber because of high moisture contents , lack of air and physiologically active defence mechanisms, but these do not appear to significantly affect the *Trichoderma* used in this experiment. Koch and Thongjiem (1989) isolated *Trichoderma* from standing trees and hence it appears that the high moisture contents found in fresh timber will not prevent *Trichoderma* from colonising the wood.

The inclusion of fresh and frozen timber showed that the differences between physiologically active and deceased timber. This appears to have made a difference to both the colonisation and subsequent increases in permeability by the *Trichoderma* isolates. In fresh timber the defence mechanisms employed by the tree may still been capable of inhibiting the growth of the invading organism and hence over the 14 weeks, the growth of the organism may have been reduced to a slower rate. *Trichoderma* was isolated from the discs cut from the fresh material (albeit at a lower frequency) but when the permeabilities of the timber were measured there was no significant increase in permeability.

With the frozen material, there was a higher incidence of *Trichoderma* being isolated from the material. This is presumably due to the defence mechanisms of the tree being disrupted by freezing, making the timber easier to colonise. This ease of colonisation may have resulted in the uninoculated frozen logs becoming contaminated with *Trichoderma* during the course of the experiment, hence fresh control logs were introduced for the permeability and moisture content determinations. The isolation of *Trichoderma* from the logs does not guarantee increases in permeability. After 9 weeks *Trichoderma* was isolated from both fresh and frozen logs but only one treatment showed significant increases in permeability. It may be

that the organism will require a longer period of time in contact with the wood to breakdown the structures reducing the permeability or that the amount of *Trichoderma* present was not great enough to breakdown these structures. The isolation of *Trichoderma* from the logs only shows that the organism has reached the sample point and not that the permeability has been increased. The positioning of the discs that were used to measure the permeability may also have affected the results. Blocks were cut from a central disc and this was likely to have been the wettest area and the area furthest from the inoculation site. Hence after 9 weeks the *Trichoderma* may not have reached this site in sufficient quantity to alter the permeability or had not had sufficient time to degrade the pit structures that limit permeability.

It should be noted that part of the observed increase in permeability could be due to natural variation, but the magnitude of increase in the frozen samples was unexpectedly high. Since freezing has no significant effect on the permeability of wood samples (3.7.3) it appears likely that these increases in permeability are due to biological action. Carey (1980) used this method for measuring permeability in unmatched wood blocks and showed significant increases in permeability of samples after exposure to biological agents. These increases were similar to those seen in this experiment.

From this experiment it is possible to see that *Trichoderma* are capable of growing through timber at high moisture contents and that there may be increases in permeability after exposure to the organism. It should also be noted that the number of replicates used in this study was low and that little work was done to examine intra- and inter-log variation.

3.9. CONCLUSIONS.

The results of the experiments in this section have shown that decalin can be used successfully to determine the permeability of different wood species and changes in the permeability after different treatments. Decalin can also be used to illustrate uptakes in different flow directions. This method should also be suitable to measure the increase of permeability of small wood blocks provided the changes in permeability are of a sufficiently high magnitude. Decalin can detect increases in permeability by *Trichoderma* isolates on Sitka spruce roundwood samples.

Chapter 4 Effect of *Trichoderma* Treatment on the Decalin uptake of Sample Wood Blocks.

4.1. Introduction

The effects of biological agents growing through wood has long been observed as fungi rapidly colonise freshly felled timber and decay wood in service. *Trichoderma* isolates are generally soil inhabiting organisms but have been shown to grow through different wood species (Spradling-Chidester, 1942: Hulme and Shields, 1972 a & b). The actions of some biological agents (including *Trichoderma*) have been shown to improve the permeability of the treated timber. Dunleavy and Fogarty (1971) showed a large increase in permeability of Sitka and Norway spruce (*Picea abies* L) after the wood had been ponded for 18 weeks. This increase in permeability was due to bacterial growth and degradation of pit membranes. Spradling-Chidester (1942) showed that *Trichoderma* would grow through Loblolly pine sapwood with only a slight reduction in the strength properties of the wood.

The aims of the following experiments were to investigate the effect of *Trichoderma* isolates selected on the basis of enzyme production (Chapter 2) on the permeability of fresh and air dried wood samples of Scots pine and Sitka spruce.

4.1.2 Methods.

Wood blocks (50 x 25 x 15mm) were cut from fresh and dried Scots pine and Sitka spruce, sapwood and heartwood. These blocks were cut to ensure that the largest tangential faces of the blocks would allow fluid uptake in a radial direction. Blocks were cut in matched pairs because of the inherent variability of wood and were numbered for identification purposes as they were cut. In total 320 matched pairs were cut from each wood species. Air dried and fresh blocks were to be treated separately. As autoclaving alters the permeability of the treated timber (Nicholas and Thomas, 1968(a)) dried wood blocks were sterilised with ethylene oxide using the following protocol:

Dried blocks were stacked in desiccators, placed in an isolation cabinet. Fifty ml of ethylene oxide was then poured into a beaker and placed along with a beaker of ultra-pure water into each desiccator. The lid was then replaced and sealed with vaseline. The outside of the desiccators and the surrounding atmosphere was sterilised by placing an extra beaker of ethylene oxide and another of ultra-pure water in the cabinet. The cabinet was then sealed and the blocks were left for 48 hours to sterilise. After the required sterilising period an extraction fan was started 30 minutes prior to opening the cabinet to exhaust the sterilant to the atmosphere. The fan was then stopped, the cabinet opened and the lids of the desiccators opened. The cabinet was then resealed and the fan restarted. The blocks were left for 48 hours to allow any excess ethylene oxide to dissipate. After 48 hours the fan was finally switched off and the cabinet opened and the desiccators again sealed. The fresh material was not sterilised as ethylene oxide sterilisation would alter the moisture content of the wood blocks; autoclaving would alter the permeability and nutrient status of the samples, and the time scale required for radiation treatment would also permit prolonged drying.

If this *Trichoderma* treatment is to be used commercially on green timber then the selected organisms must be able to grow in the presence of other wood resident organisms in the first instance, hence the fresh blocks were placed into inoculation jars immediately after cutting and weighing.

The medium used in this experiment was a minimal medium based on that used by Huttermann and Volger(1973) (see Chapter 2). Inoculation jars were prepared by adding 200 ml of medium and 1% agar to each jar, sealed and autoclaved at 121^{o}C for 20 minutes. Once cool, jars were inoculated with one of the five previously selected *Trichoderma* isolates (see section 2.4) and incubated at either 22 or 25^{o}C as appropriate until mycelia had totally colonised the agar surface. Once colonised a sterile piece of plastic matting was placed on top

of the mycelial mat, to keep the wood blocks above the surface of the agar and prevent uptake of moisture and nutrients from the medium. The jars were then ready for the addition of the sample blocks.

Blocks were weighed prior to incubation and one of each matched pair was placed in the inoculation jars with the *Trichoderma* isolates. Four replicate blocks were tested in each jar. The other half of the pair was immediately dried down at 103°C to constant weight. Blocks were incubated with the selected *Trichoderma* isolates for 5 or 10 weeks. On completion of the incubation the blocks were removed from the jars and all surface mycelia were cleaned off before the blocks were weighed and dried down to constant weight at 103°C.

The permeability of the matched pairs of wood blocks was then measured by dipping the blocks in decalin for 10 seconds as described in section 3.2.2. Care was taken to ensure that the orientation of the blocks always remained the same whilst being dipped. The results for individual blocks were then compared with the corresponding matched pair and the pooled results analysed using the General Linear Model (GLM). The GLM is statistical test that permits data to be analysed in a manner similar to the Analysis of Variance (ANOVA). The GLM differs from ANOVA by permitting a multi-variate analysis of data. rather than a one or two way analysis (Wetherill, 1981).

4.1.3 Results

The moisture contents of the wood blocks can be seen in Table 4.1

Fresh/Dried	Wood type	Initial moisture (%) (Dry Wt basis)	St. Dev	Final moisture (%) (Dry Wt basis)	St. Dev
Fresh	Scots pine sapwood	151	30	122	81
	Scots pine heartwood	31	10	56	49
	Sitka spruce sapwood	240	45	215	86
	Sitka spruce heartwood	85	28	92	59
Dried	Scots pine sapwood	7	1.4	32	15
	Scots pine heartwood	6	1.4	31	19
	Sitka spruce sapwood	6	1.7	27	11
	Sitka spruce heartwood	6	0.9	44	25

Table 4.1 Mean moisture contents of wood blocks before and after incubation with *Trichoderma* isolates.

The results in table 4.1 show that with the fresh timber most samples start well above fibre saturation point, the exception was the pine heartwood which as expected was at approximately fibre saturation point. Mean moisture contents remained fairly constant during the incubation, but within the experiment there was a huge variation in the moisture content as some blocks dried down to below fibre saturation point and hence may have discouraged colonisation of the blocks. Dried wood blocks all started below fibre saturation point and during the course of the experiment absorbed some moisture to finish the experiment at or close to the fibre

saturation point. The wetting up of the blocks would take time and hence the blocks may not have been effectively colonised by the *Trichoderma* isolates. Despite these moisture imbalances and their possible effects on colonisation some of the isolates still showed significant increases in permeability and a summary of these results can be seen in Table 4.2..

		Fresh				Dried			
		Scots pine		Sitka spruce		Scots pine		Sitka spruce	
Organism/	Time (wks)	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood
T. aureoviride SIWT.1	5				*		*	*	
	10	*		*			*		*
T. polysporum IMI 206039	5	*					*		
	10	*					*		*
T. pseudokoningii SIWT 51	5	*				*	*		*
	10					*			
T. pseudokoningii SIWT 64	5						*	*	*
	10	*				*	*		*
T. viride SIWT 70	5					*	*	*	*
	10	*		*	*	*			*

Table 4.2. Summary of the effects on permeability of *Trichoderma* isolates on fresh and dried Scots pine and Sitka spruce sapwood and heartwood. N.B. "*" represents a significant increase in permeability at the 95% confidence intervals

The results of the statistical analysis show that most significant permeability increases in fresh material were observed in the sapwood material particularly Scots pine. This may be due to the presence of compounds in the heartwood that prevent effective colonisation by the *Trichoderma* isolates or that the changes in permeability are too small to be effectively detected by the decalin method. In dried samples the heartwood material showed more significant increases in permeability which again may be related to the extractive content of the heartwood. As heartwood is dried, extractives may be removed from the timber allowing easier colonisation and effective increases in permeability. There were still significant increases in permeability observed in some sapwood samples.

The mean % increase in decalin uptake between matched pairs can be seen in figures 4.1-4.8 for which the following key represents the identity of the *Trichoderma* isolate.

Key: "T1.5" represents organism *T. aureoviride* SIWT.1 after 5 weeks incubation.
"T1.10" represents organism *T. aureoviride* SIWT.1 after 10 weeks incubation
"T2.5" represents organism *T. polysporum* IMI 206039 after 5 weeks incubation.
"T2.10" represents organism *T. polysporum* IMI 206039 after 10 weeks incubation.
"T3.5" represents organism *T. pseudokoningii* SIWT 51 after 5 weeks incubation.
"T3.10" represents organism *T. pseudokoningii* SIWT 51 after 10 weeks incubation.
"T4.5" represents organism *T. pseudokoningii* SIWT 64 after 5 weeks incubation.
"T4.10" represents organism *T. pseudokoningii* SIWT 64 after 10 weeks incubation.
"T5.5" represents organism *T. viride* SIWT 70 after 5 weeks incubation.
"T5.10" represents organism *T. viride* SIWT 70 after 10 weeks incubation.

The results of the mean % increase in decalin uptake by fresh Scots pine sapwood can be seen in Figure 4.1

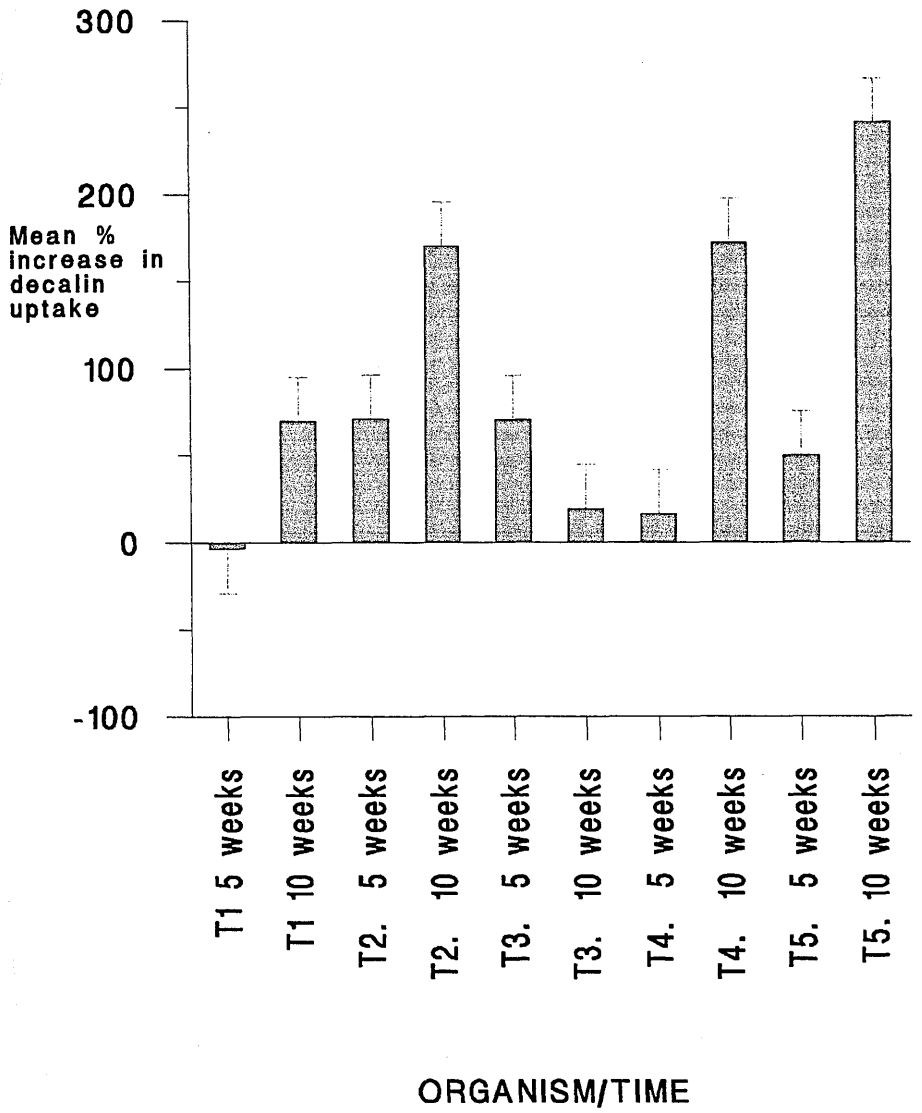


Figure 4.1 Mean % increase in decalin uptake by fresh Scots pine sapwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

These results correspond well with the results in table 4.2. In all but one organism increases in decalin uptake are greater after 10 weeks compared with 5 weeks incubation.

The results of the mean % increase in decalin uptake by fresh Scots pine heartwood can be seen in Figure 4.2

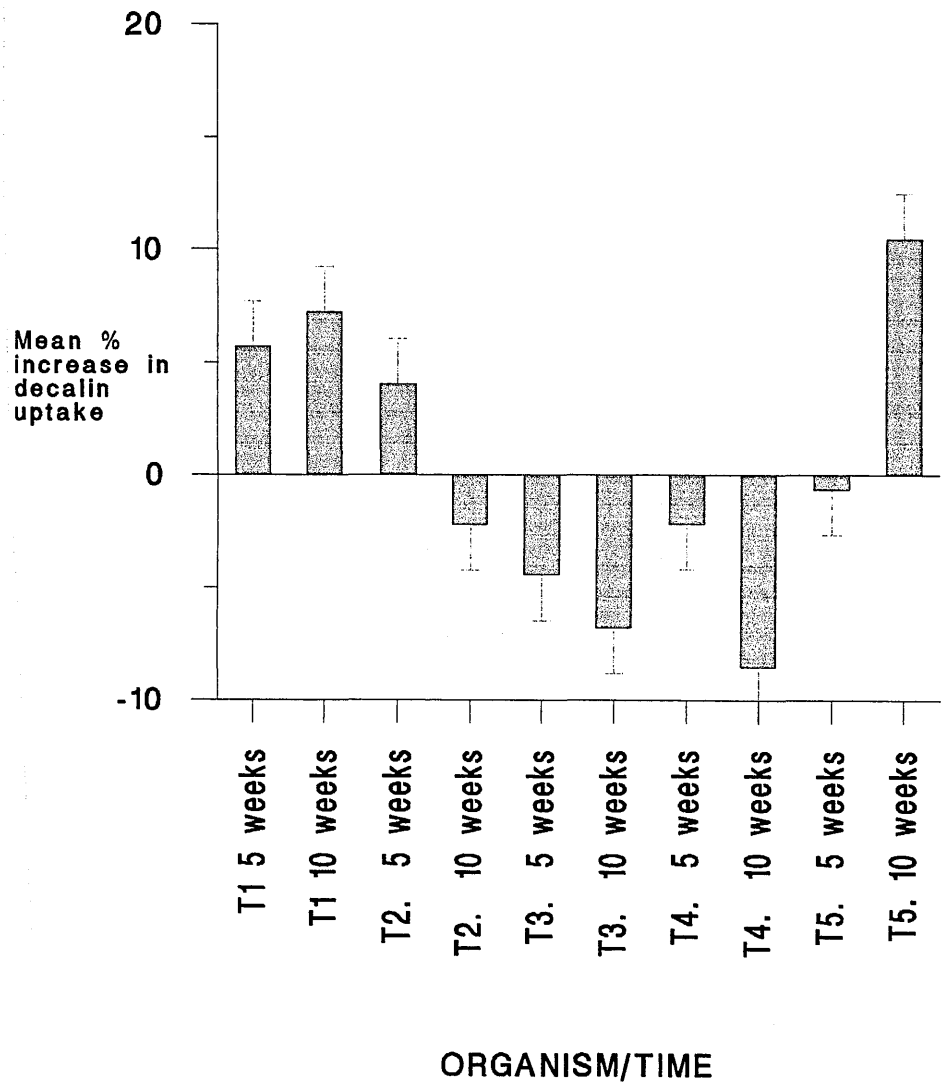


Figure 4.2 Mean % increase in decalin uptake by fresh Scots pine heartwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

These clearly indicate only minor changes in permeability enhancement none of which are significant as shown in table 4.2.

The results of the mean % increase in decalin uptake by fresh Sitka spruce sapwood can be seen in Figure 4.3.

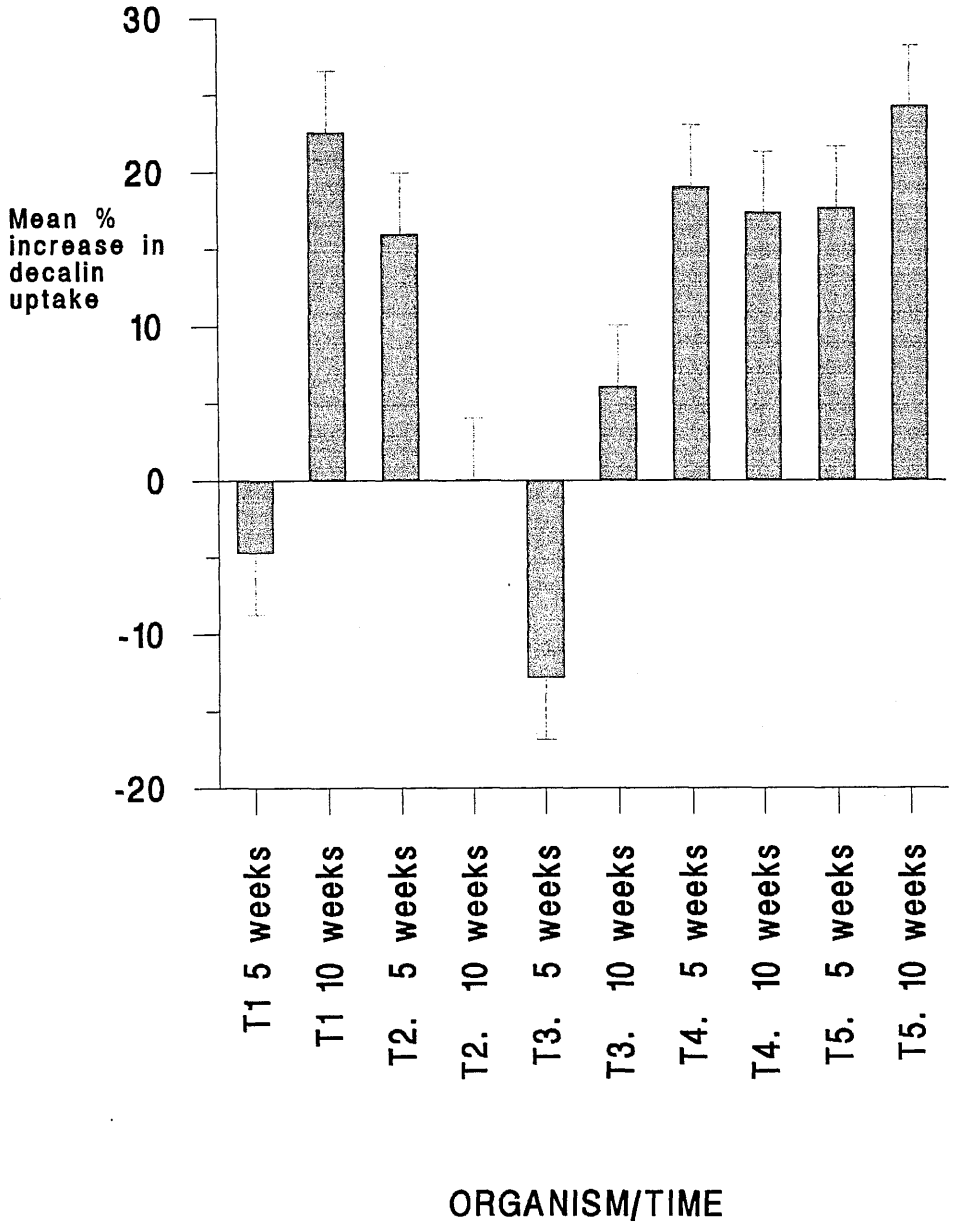


Figure 4.3 Mean % increase in decalin uptake by fresh Sitka spruce sapwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

Only two isolates showed significant increases in permeability *Trichoderma aureoviride* (T1) and *Trichoderma viride* (SIWT 70) (T5) after 10 weeks incubation.

The results of the mean % increase in decalin uptake by fresh Sitka spruce heartwood can be seen in Figure 4.4

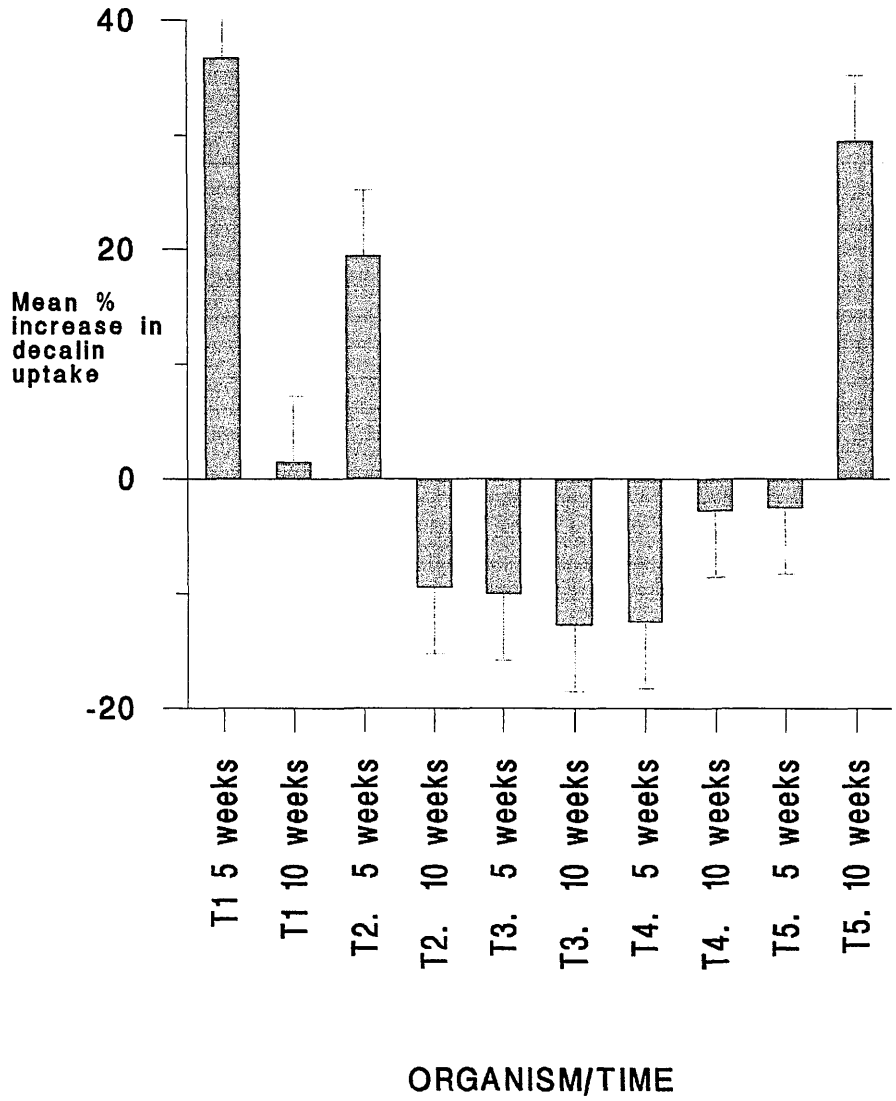


Figure 4.4 Mean % increase in decalin uptake by fresh Sitka spruce heartwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

Only two isolates showed significant increases in permeability *Trichoderma aureoviride* and *Trichoderma viride* (SIWT 70) with no apparent correlation between incubation time and improved ability of the organisms to increase the permeability of the wood samples.

The results of the mean % increase in decalin uptake by dried Scots pine sapwood can be seen in Figure 4.5

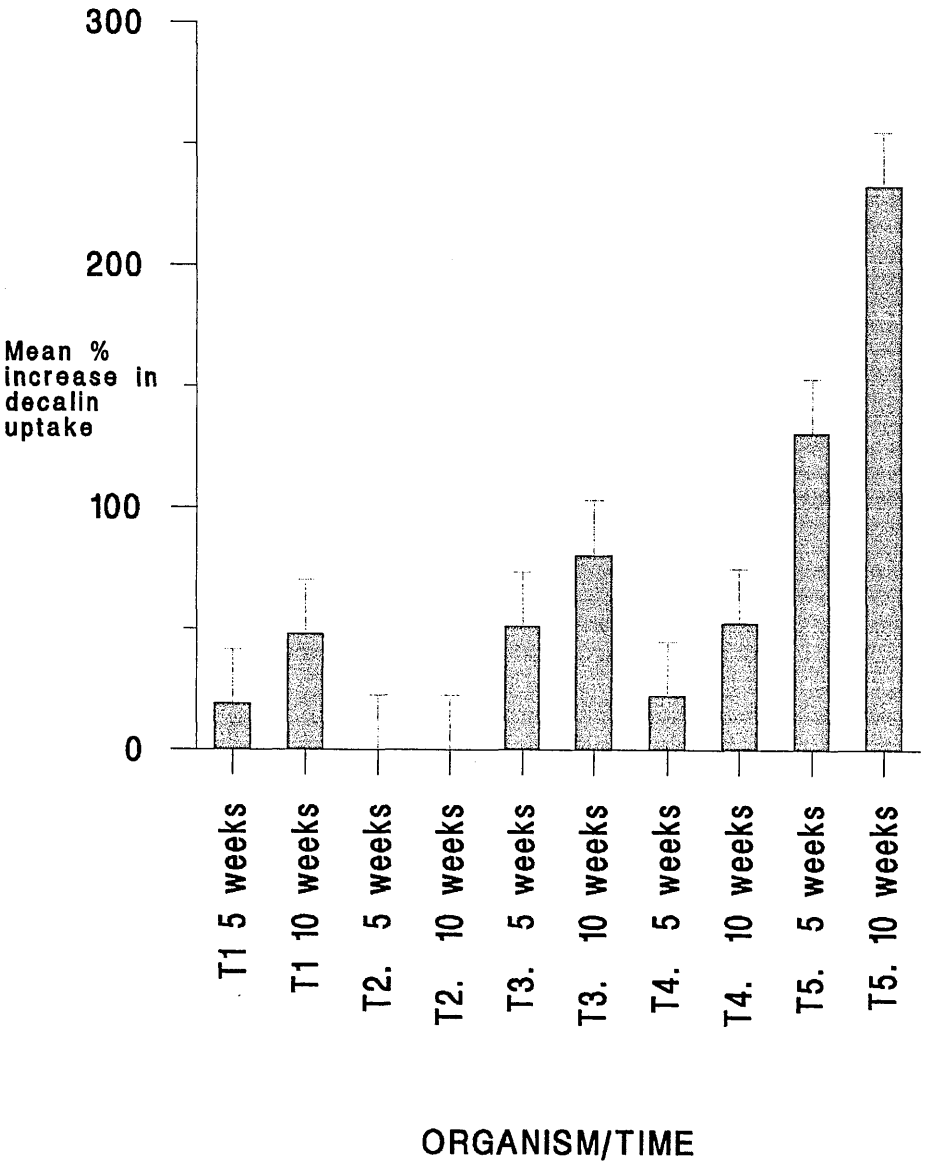


Figure 4.5 Mean % increase in decalin uptake by dried Scots pine sapwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

In all cases except *T.polysporum* (T2) permeability increases improved between 5 and 10 week incubation period however only *Trichoderma pseudokoningii* 51 (T3), *Trichoderma pseudokoningii* 64 (T4) and *Trichoderma viride* 70 (T5) were statistically greater than control material.

The results of the mean % increase in decalin uptake by dried Scots pine heartwood can be seen in Figure 4.6

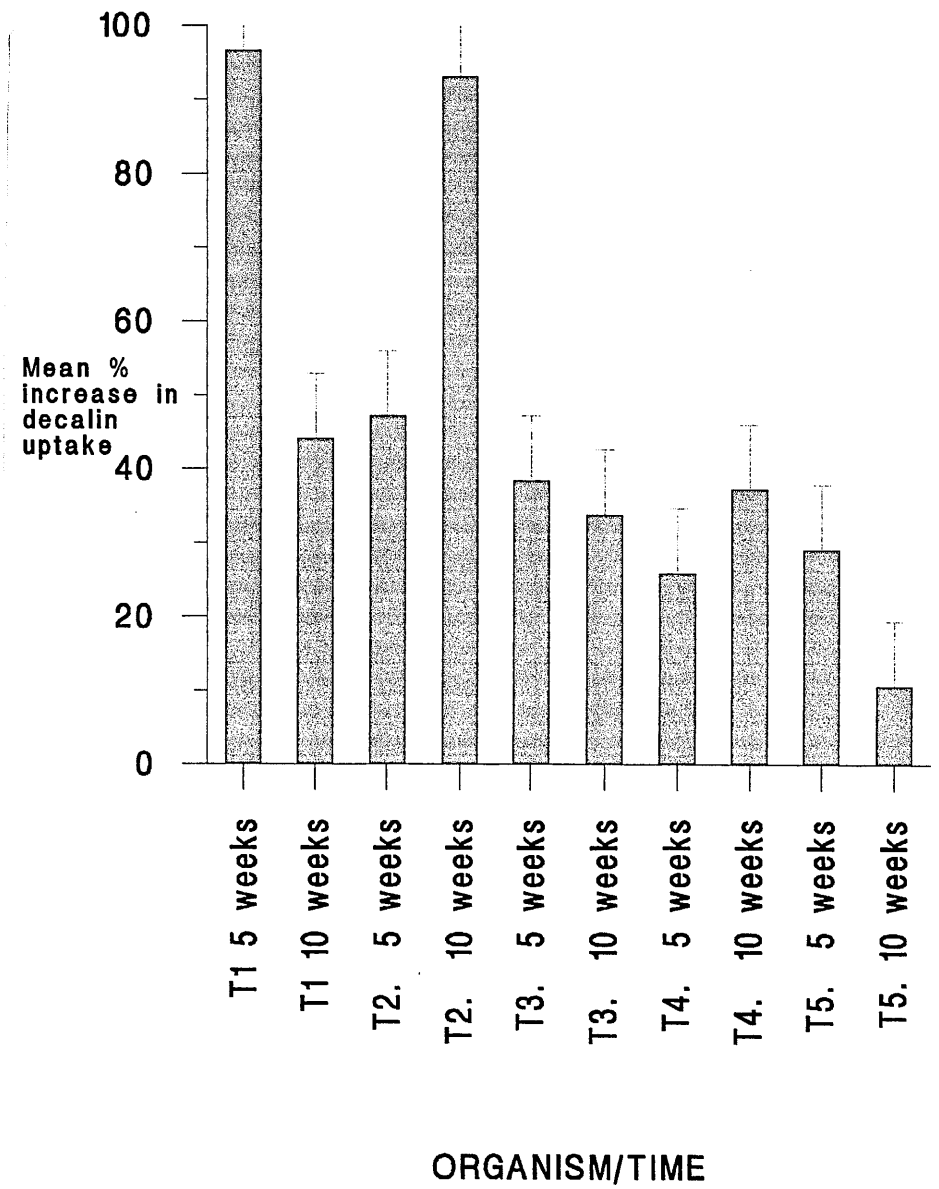


Figure 4.6 Mean % increase in decalin uptake by dried Scots pine heartwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

All samples except *Trichoderma pseudokoningii* 51 after 10 weeks and *Trichoderma viride* 70 after 10 weeks were significantly different from the control material.

The results of the mean % increase in decalin uptake by dried Sitka spruce sapwood can be seen in Figure 4.7.

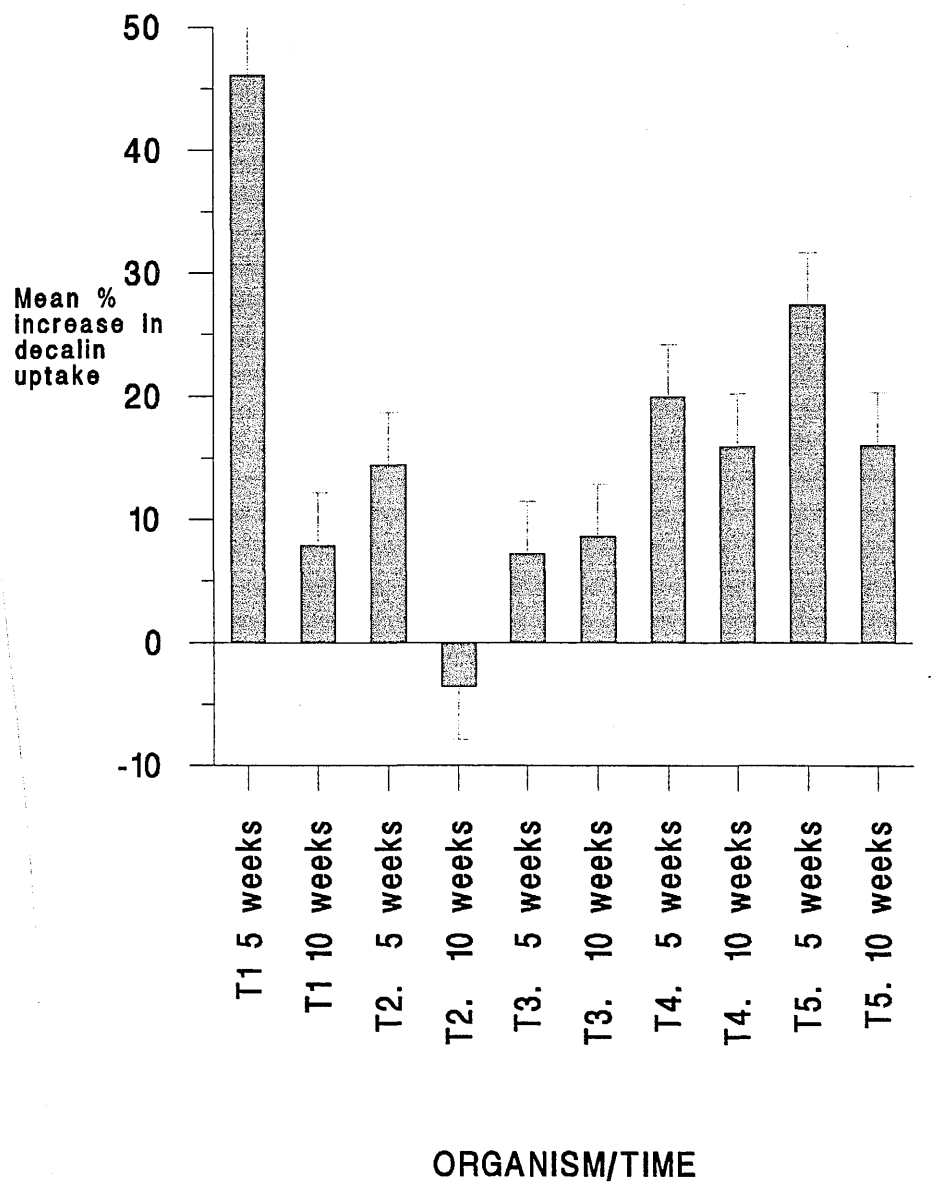


Figure 4.7 Mean % increase in decalin uptake by dried Sitka spruce sapwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

Although most isolates showed increases in permeability only three organisms showed any significant increases in permeability over the control material and all were after 5 weeks incubation period, the isolates concerned were *Trichoderma pseudokoningii* SIWT.64, *Trichoderma aureoviride* SIWT.1, *and Trichoderma viride* SIWT.70.

The results of the mean % increase in decalin uptake by dried Sitka spruce heartwood can be seen in Figure 4.8

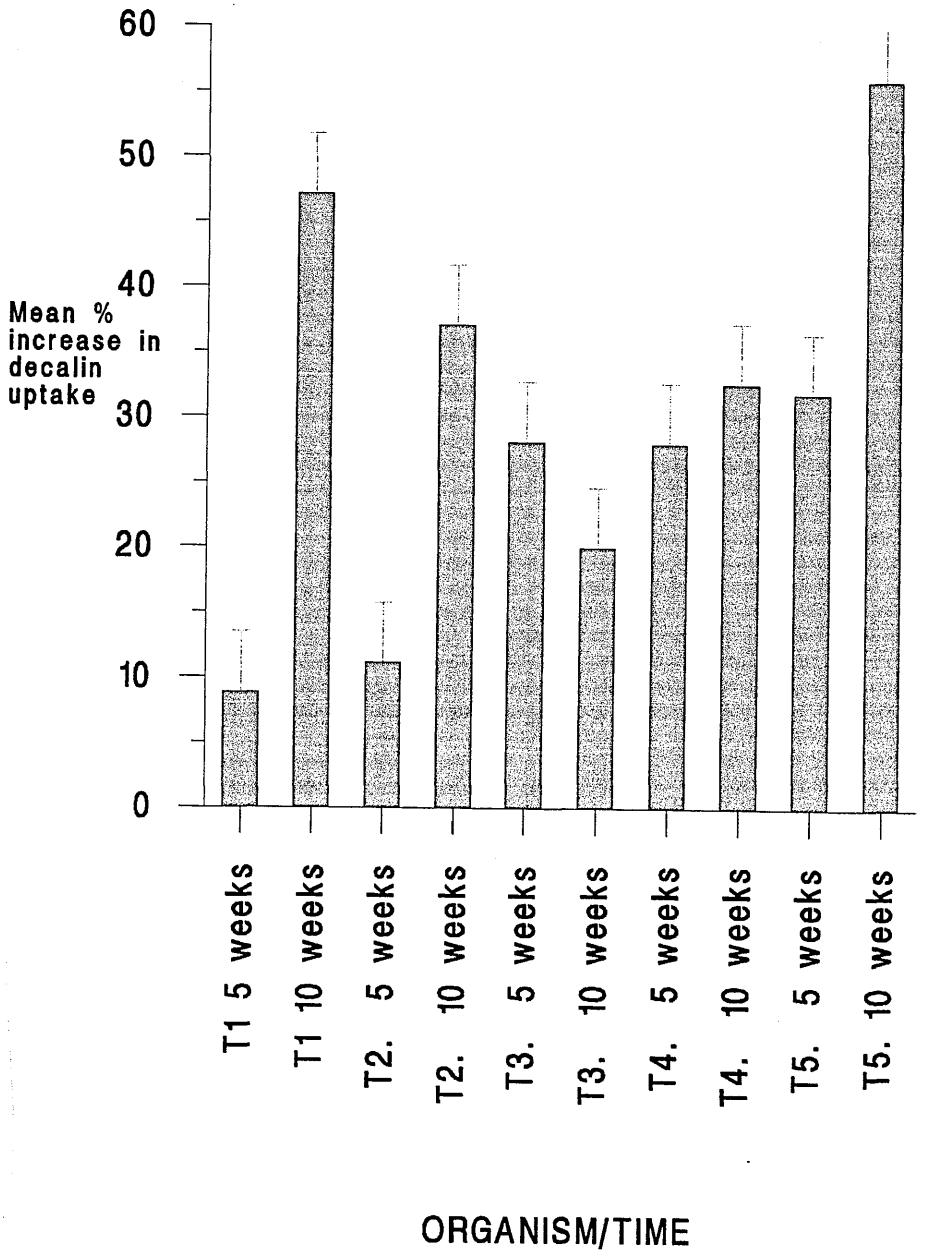


Figure 4.8 Mean % increase in decalin uptake by dried Sitka spruce heartwood after incubation with selected *Trichoderma* isolates. N.B. Error bars were calculated as the standard error of the data set.

All isolates showed a positive increase in permeability with only three samples showing no significant increase in permeability over control material despite showing a change in permeability (*Trichoderma aureoviride* SIWT.1(5 weeks), *Trichoderma pseudokoningii* SIWT.51 (10 weeks), and *Trichoderma polysporum* (5 weeks)).

The results in figures 4.1-4.8 show the differences between the increases in decalin uptake and some of the variation that occurs between the *Trichoderma* isolates. There will also be the factor of the desiccation of some of the treatments included in these results which may have resulted in a lower uptake of decalin as growth and colonisation by the *Trichoderma* isolates will be poorer.

4.1.4.Discussion

The results show that there is an a significant increase in the permeability of many of the wood blocks treated with *Trichoderma*. Permeability increases in individual matched pairs showed large variation in permeability even within treatment groups. This may simply indicate that matched pairs of blocks had come from different parts of the log. Hence the results were expressed as a mean % increase in decalin uptake for the four replicates.
It was noted that there was poor contact between the wood blocks and the mycelial mat in some jars and hence colonisation of the blocks would be poor. During the experiment there was also some evidence of the agar shrinking during the incubation and lowering the mycelia away from the wood blocks permitting the blocks to dry. Despite these colonisation problems some of the isolates caused significant increases to the permeability of the wood blocks. In those jars that had poor colonisation the *Trichoderma* may be capable of improving the permeability of the blocks but because of the colonisation problems may have been prevented from doing so. It is clear from this experiment that there are large differences between the abilities of the isolates to improve the permeability of different wood types.

Isolates *T. aureoviride* SIWT.1 and *T. viride* SIWT 70 showed the largest number of statistically significant increases in permeability across the range of wood types tested and an ability to colonise wood blocks cut from both species.
Heartwood material is physiologically distinct from the outer sapwood and hence the effects of the *Trichoderma* isolates on this material may be different. In pine heartwood extraneous materials are deposited as the wood differentiates during growth of the tree

which gives rise to the natural durability of the timber. The results of this experiment appear to show that *Trichoderma* can grow through the dried heartwood material and significantly improve its permeability more easily than in the fresh material. This may be due to some of these extraneous compounds being volatilised or degraded as the timber dries.

As the results from this experiment were affected by the conditions experienced during the incubation it was decided to repeat the experiment using a slightly different methodology (section 4.2).

4.2. REPETITION OF INVESTIGATION INTO THE EFFECT OF *TRICHODERMA* TREATMENT ON THE UPTAKE OF DECALIN

4.2.1. Introduction

Earlier work (Section 4.1) has indicated that *Trichoderma* isolates will improve the permeability of wood samples. Due to experimental difficulties on maintaining contact between the fungal mycelial mat and the wood blocks, experiment (4.1) was repeated to establish that when given suitable conditions the selected *Trichoderma* isolates could increase the permeability of different samples of Scots pine and Sitka spruce.

4.2.2. Methods

Isolates selected for these studies had previously been screened for the production of amylase, pectinase and cellulase. In the previous permeability experiment (section 4.1) only those isolates showing good enzyme production were exposed to the different wood species. For this repeated experiment these were supplemented with a control isolate (*Trichoderma viride* SIWT 100) which had shown little ability to produce selected enzymes in the earlier screening.

Blocks were cut in matched pairs from fresh and dried Scots pine and Sitka spruce sapwood and heartwood. These blocks were smaller in size (50 x 20 x 15mm) to prevent wedging in the jar and to ensure continuous contact with fungal mycelia throughout the experiment. Dried blocks were sterilised using ethylene oxide (section 4.1.2) before being submerged in sterile distilled water for 24 hours to raise their moisture content. Again fresh wood was not sterilised prior to exposure to the test fungi . Incubation jars were prepared as before but were not inoculated prior to the addition of the plastic matting onto the sterile agar.

Four blocks were prepared per treatment, weighed and asceptically placed upon sterile plastic matting in each incubation jar. The jars were then inoculated by spraying a spore suspension (approx. 10^6 spores per ml) onto the blocks and agar. The jars were then sealed

and incubated at 22 or 25°C for 5 or 10 weeks. On completion of this incubation the wood samples were treated in the same manner as those used in the earlier experiment.

4.2.3. Results

Three different representations of the data have been used:

1) Gross increase in decalin uptake:

Weight (g) decalin taken up by *Trichoderma* treated block - Weight (g) decalin taken up by matched control.

2) Mean increase in % uptake of decalin:

Calculated as follows

% increase in decalin uptake in *Trichoderma* treated block* - % increase in decalin uptake in corresponding control block*.

NB * Percentage increase calculated on original dry weight of block prior to treatment.

3) Mean percentage increase in uptake (%) of decalin. Calculated as follows

result of determination 2) divided by % uptake for control block.

See appendix 4 for worked examples

The results of the decalin uptake by treated and control wood blocks were analysed as described in Section 4.2.3. In the first instance the gross increase in decalin uptake was measured and the results analysed using the General Linear Model. A summary of these results of this can be seen in Table 4.3.

		Fresh				Dried			
		Scots pine		Sitka spruce		Scots pine		Sitka spruce	
Organism/Time	Time	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood
T. aureoviride SIWT.1	5	*	*			*			
	10		*	*		*		*	
T. polysporum IMI 206039	5								
	10	*				*			
T. pseudokoningii SIWT 51	5								
	10					*			
T. pseudokoningii SIWT 64	5								
	10	*		*		*			
T. viride SIWT 70	5					*			
	10	*	*	*		*		*	
T. viride SIWT 100	5					*			
	10					*			

Table 4.3. Summary of the effects on mean decalin uptake by fresh and dried Scots pine and Sitka spruce sapwood and heartwood after exposure to different *Trichoderma* isolates. N.B. "*" represents a significant increase in permeability at the 95% confidence intervals

Table 4.3 highlights those treatments that showed a significant increase in the uptake of decalin. It may be possible that only treatments with large increases in decalin uptake would be identified through this analysis. Blocks that had lower uptakes in the matched control block may not increase by a large enough magnitude to be detected by this analysis. Hence the changes in uptake were calculated and analysed as the mean % increase in permeability. The results for fresh and dried Scots pine and Sitka spruce sapwood and heartwood can be seen in figures 4.10 -4.17 for which the following key should be used.

Key: "T1.5" represents organism *T. aureoviride* SIWT.1 after 5 weeks incubation.
"T1.10" represents organism *T. aureoviride* SIWT.1 after 10 weeks incubation
"T2.5" represents organism *T. polysporum* IMI 206039 after 5 weeks incubation.
"T2.10" represents organism *T. polysporum* IMI 206039 after 10 weeks incubation.
"T3.5" represents organism *T. pseudokoningii* SIWT 51 after 5 weeks incubation.
"T3.10" represents organism *T. pseudokoningii* SIWT 51 after 10 weeks incubation.
"T4.5" represents organism *T. pseudokoningii* SIWT 64 after 5 weeks incubation.
"T4.10" represents organism *T. pseudokoningii* SIWT 64 after 10 weeks incubation.
"T5.5" represents organism *T. viride* SIWT 70 after 5 weeks incubation.
"T5.10" represents organism *T. viride* SIWT 70 after 10 weeks incubation.
"T6.5" represents organism *T. viride* SIWT 100 after 5 weeks incubation.
"T6.10" represents organism *T. viride* SIWT 100 after 10 weeks incubation.

The results (represented as mean increase in % uptake of decalin) produced by dipping fresh Scots pine sapwood after 5 and 10 weeks exposure to different *Trichoderma* isolates can be seen in figure 4.9.

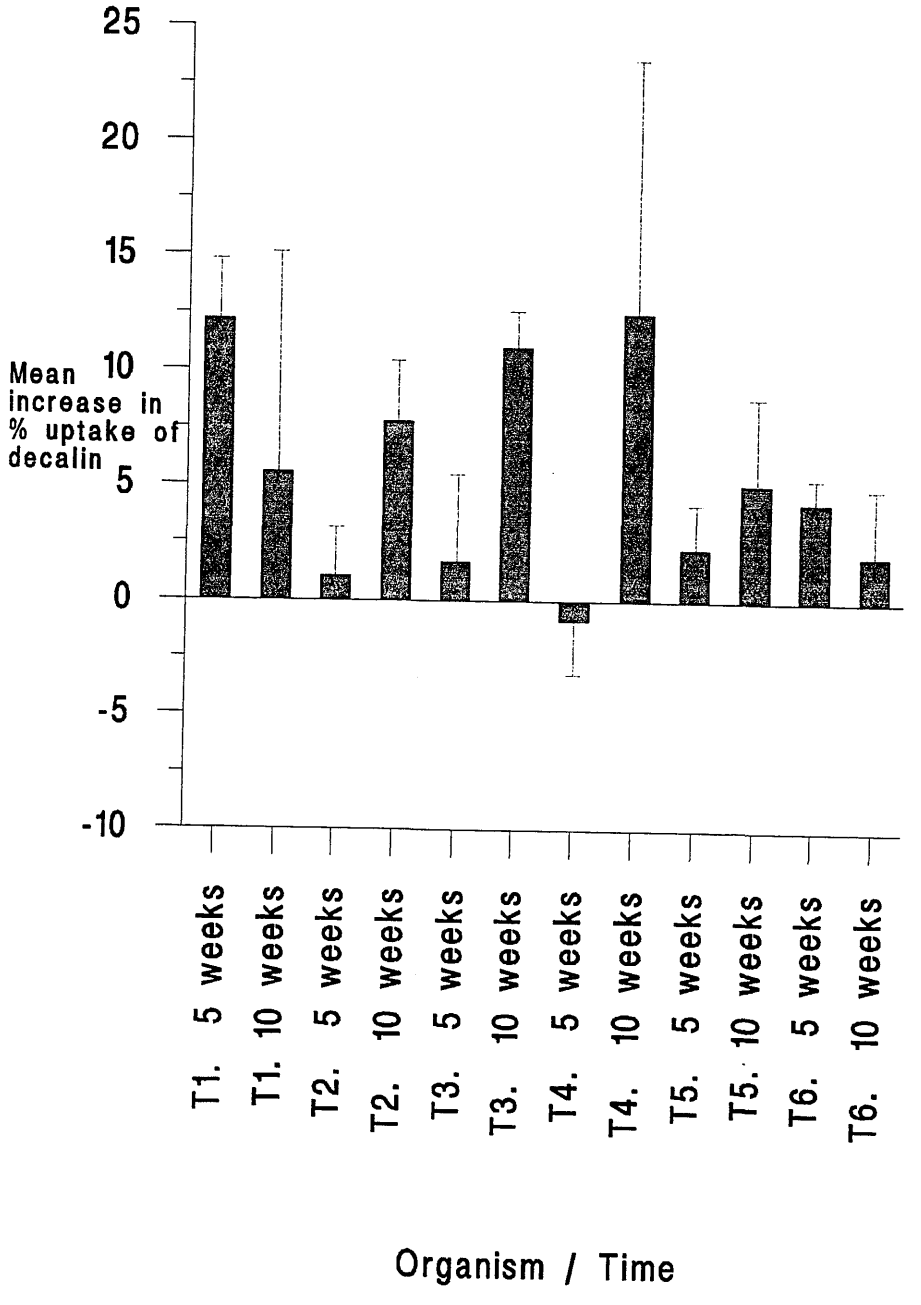


Figure 4.9. Mean increase in % uptake of decalin by fresh Scots pine sapwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

It is noticeable for all samples that there is large variability between replicates as indicated by large standard deviations.

The results of mean increase in % uptake of decalin by dried Scots pine sapwood can be seen in Figure 4.10.

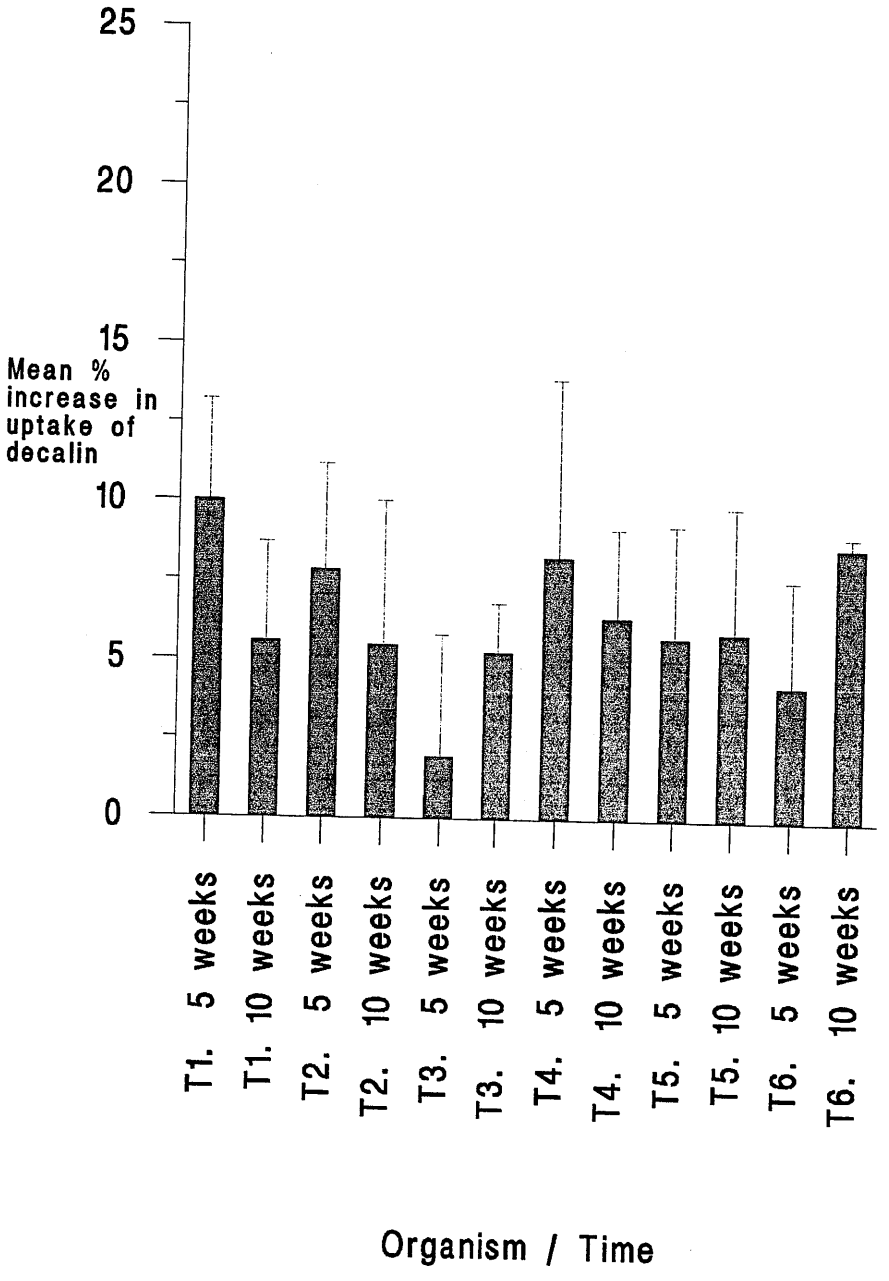


Figure 4.10 Mean increase in % uptake of decalin by dried Scots pine sapwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

Once again all blocks showed increases in % uptake of decalin however with little obvious effects of increased incubation.

The results of mean increase in % uptake of decalin by fresh Scots pine heartwood can be seen in Figure 4.11.

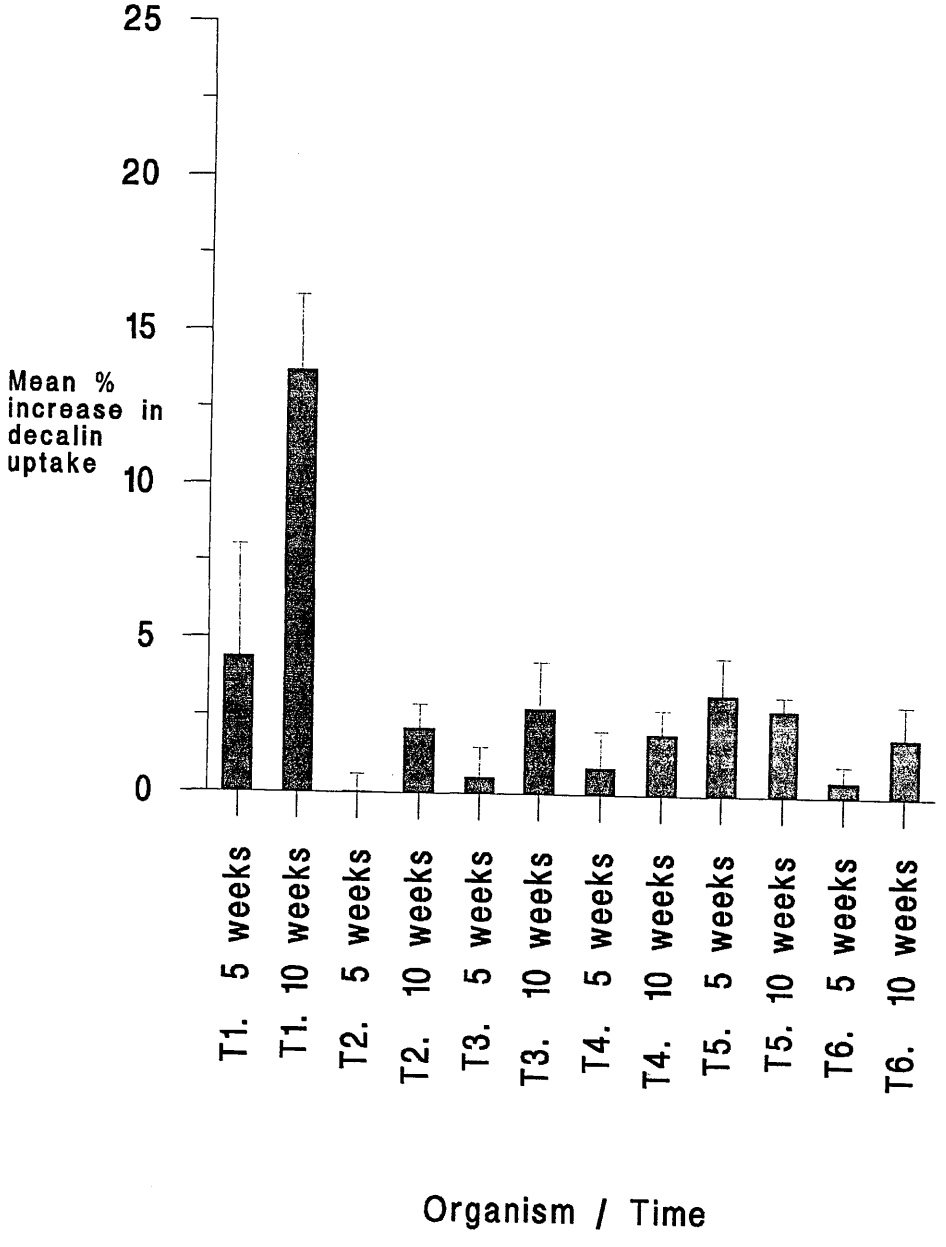


Figure 4.11. Mean increase in % uptake of decalin by fresh Scots pine heartwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

The levels of increase are noticeably smaller than those for either dried or fresh Scots pine sapwood samples. In most cases however there is evidence of increased uptake levels in samples incubated for 10 weeks.

The results of mean increase in % uptake of decalin by dried Scots pine heartwood can be seen in Figure 4.12

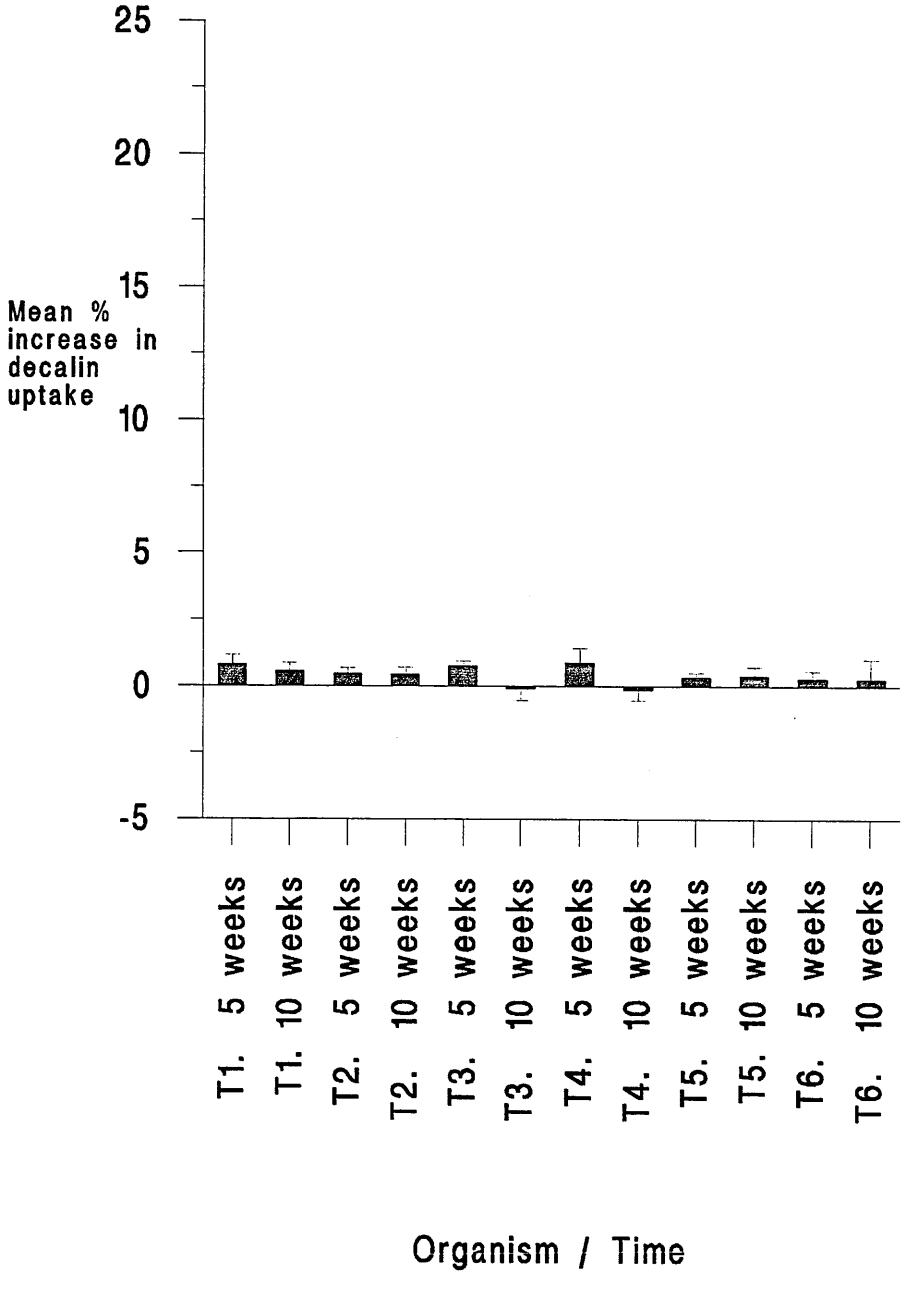


Figure 4.12. Mean increase in % uptake of decalin by dried Scots pine heartwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

It is obvious that levels are much lower that those even in fresh Scots pine heartwood.

The results of mean increase in % uptake of decalin by fresh Sitka spruce sapwood can be seen in Figure 4.13.

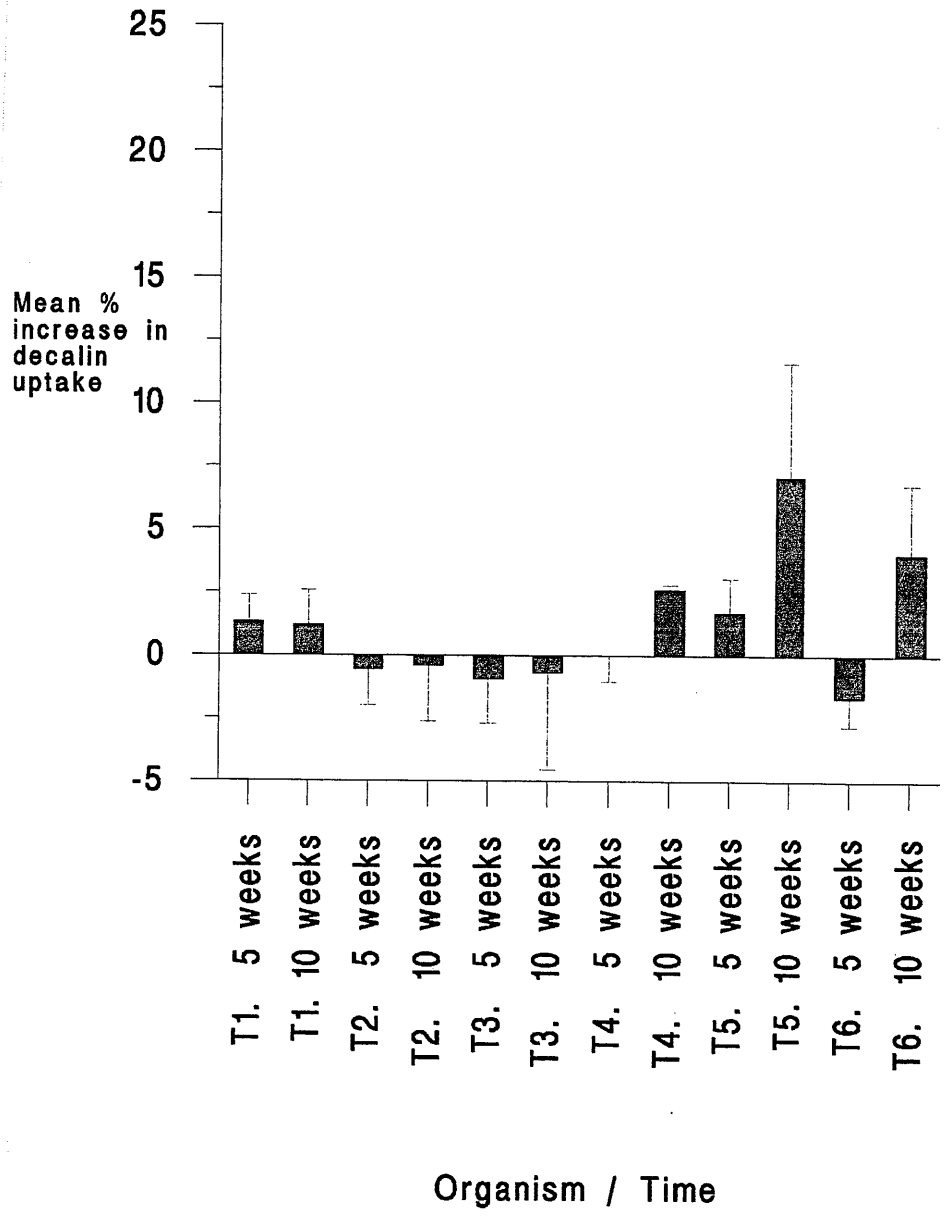


Figure 4.13. Mean increase in % uptake of decalin by fresh Sitka spruce sapwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

Levels of permeability increase observed with spruce sapwood are lower than pine sapwood for most treatments.

The results of mean increase in % uptake of decalin by dried Sitka spruce sapwood can be seen in Figure 4.14.

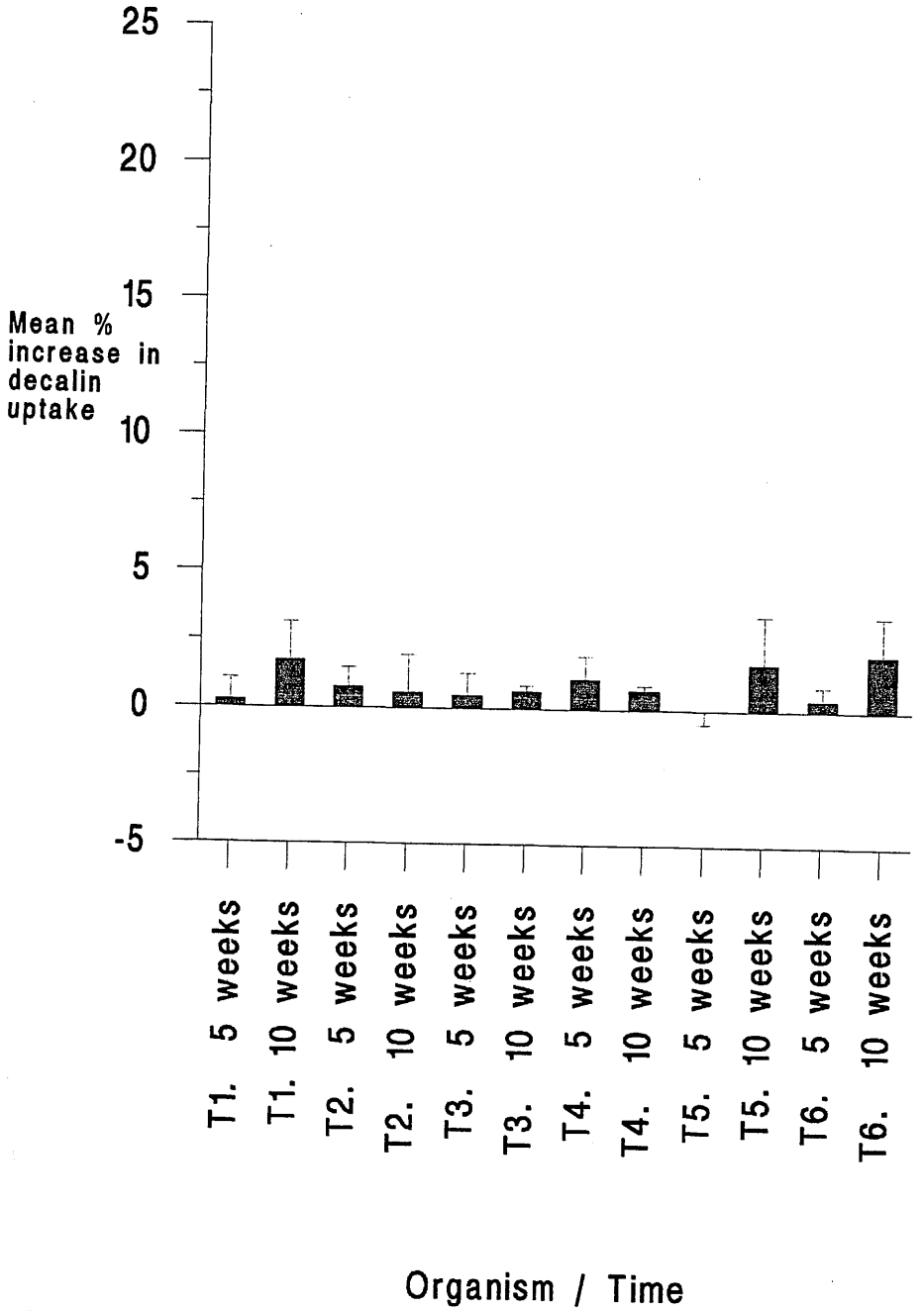


Figure 4.14 Mean increase in % uptake of decalin by dried Sitka spruce sapwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

Most treatments showed an increase in permeability although uptake levels were considerably lower than in fresh material, because of variation in uptake few samples showed significant increases.

The results of the mean increase in % uptake of decalin by fresh Sitka spruce heartwood can be seen in Figure 4.15.

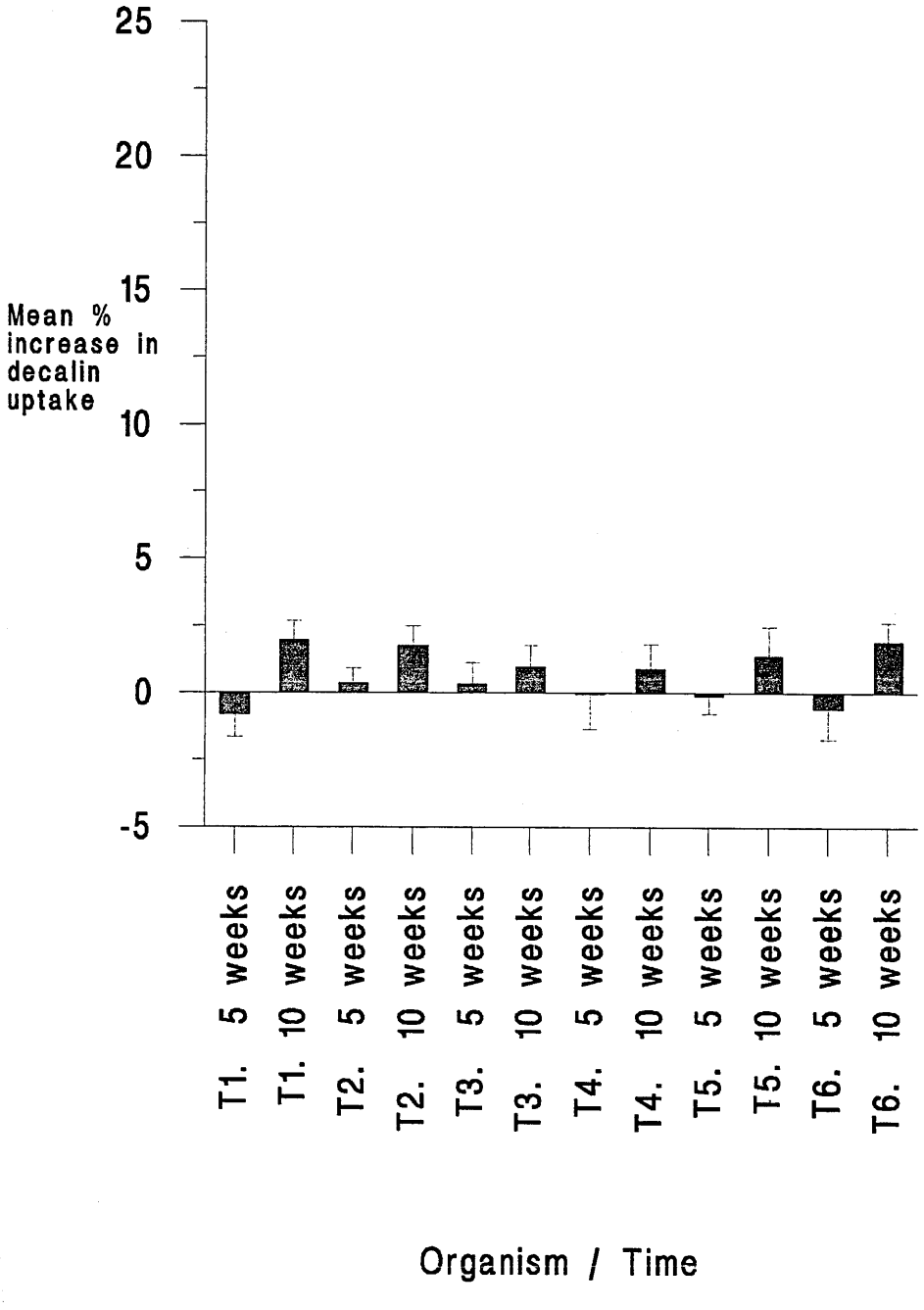


Figure 4.15. Mean increase in % uptake of decalin by fresh Sitka spruce heartwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

Fresh heartwood material showed increases in uptake in all samples tested after 10 weeks incubation. although levels were again lower than pine sapwood

The results of mean increase in % uptake of decalin by dried Sitka spruce heartwood can be seen in Figure 4.16

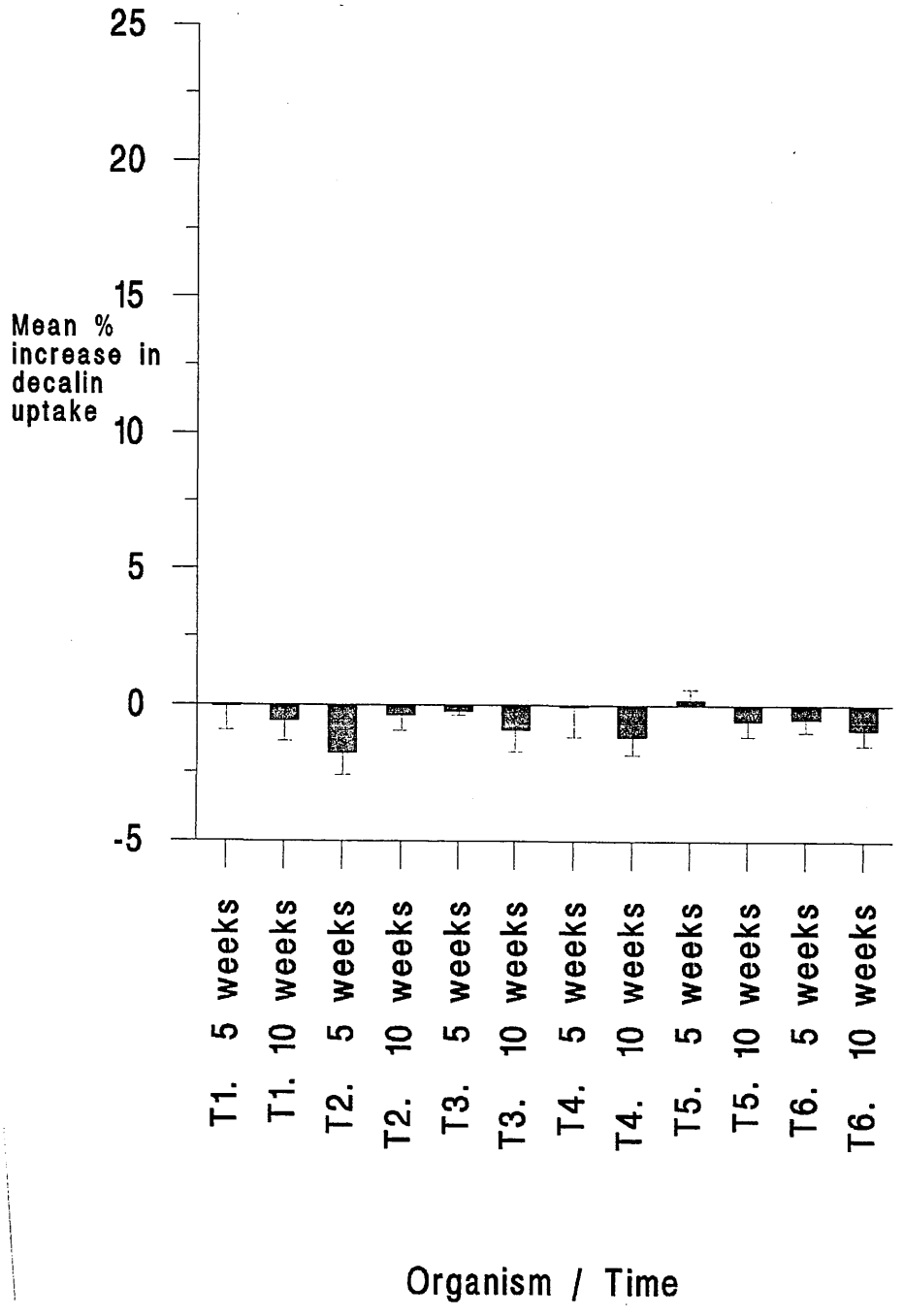


Figure 4.16 Mean increase in % uptake of decalin by dried Sitka spruce heartwood after incubation with selected *Trichoderma* isolates. N.B Error bars were calculated as the standard deviation of the data set.

Few samples showed any increase in decalin uptake after *Trichoderma* treatment and all changes were relatively small.

The results in figures 4.9- 4.16 show the mean increase in decalin uptake(%) by wood blocks cut from Scots pine and Sitka spruce. The results illustrate the variability of *Trichoderma* in their ability to improve the permeability of different wood types. It is clear that the mean % increase in decalin uptake in spruce is generally lower than that recorded in pine with indeed little change occurring in any of the dried Sitka spruce heartwood samples.

These results are based only on the increase in uptake of decalin between the pairs of wood blocks. The natural variability in permeability of blocks from different regions of the logs however may mask increases in permeability produced by the *Trichoderma* treatment. The data was therefore analysed for changes in mean % increase in uptake (%) of decalin using GLM . The results of this analysis can be seen in tables 4.4, 4.5 and 4.6.

		Fresh				Dried			
		Scots pine		Sitka spruce		Scots pine		Sitka spruce	
Organism / Time	Time	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood	Sapwood	Heartwood
T. aureoviride SIWT.1	5	*	*			*	*		
	10	*	*	*	*	*	*	*	
T. polysporum IMI 206039	5					*	*		
	10	*	*		*	*	*		
T. pseudokoningii SIWT 51	5						*		
	10	*	*			*			
T. pseudokoningii SIWT 64	5					*	*	*	
	10	*	*	*		*			
T. viride SIWT 70	5	*	*			*	*		
	10	*	*	*	*	*	*	*	
T. viride SIWT 100	5	*				*			
	10			*	*	*	*		

Table 4.4. Summary of the effects on mean % increase in uptake % of decalin by fresh and dried Scots pine and Sitka spruce sapwood and heartwood after exposure to different *Trichoderma* isolates. N.B. "*" represents a significant increase in permeability at the 95% confidence intervals. The actual values of the mean % increases in decalin uptake (%) for Scots pine can be seen in Table 4.5. It is apparent that the longer incubation time has resulted in improved permeability for most of the isolates in the different wood types.

		SCOTS PINE							
		Sapwood				Heartwood			
		Fresh		Dried		Fresh		Dried	
Organism	time (weeks)	Mean	Std	Mean	Std	Mean	Std	Mean	Std
T. aureoviride SIWT.1	5	94.06	20.98	112.92	69.67	80.39	83.20	68.50	32.06
	10	44.29	75.76	50.69	29.70	219.17	57.64	47.03	33.44
T. polysporum IMI 206039	5	7.01	15.05	66.21	30.98	1.17	7.71	35.07	15.04
	10	58.08	21.96	47.72	36.00	32.96	16.47	33.84	19.16
T. pseudokoningii SIWT 51	5	11.50	26.23	19.65	29.66	9.63	14.67	54.08	8.52
	10	69.56	16.53	45.13	13.95	37.91	22.01	-2.68	27.00
T. pseudokoningii SIWT 64	5	-6.25	16.82	69.74	40.96	10.48	13.42	76.69	53.65
	10	89.45	83.81	53.89	27.01	26.27	12.03	-5.81	15.79
T. viride SIWT 70	5	17.26	13.86	49.50	27.76	43.07	20.59	25.43	17.41
	10	36.07	26.66	42.99	32.46	43.21	11.54	30.54	25.04
T. viride SIWT 100	5	28.61	6.43	31.43	30.07	6.76	7.91	19.36	20.88
	10	13.75	20.20	67.05	3.19	20.25	10.20	28.99	48.80

Table 4.5. Mean % increase in % uptake of decalin by fresh and dried Scots pine wood blocks after incubation with selected *Trichoderma* isolates for 5 or 10 weeks.

The actual values of the mean % increases in decalin uptake (%) for Sitka spruce can be seen in Table 4.6. It is clear that far fewer significant increases are produced in Sitka spruce than Scots pine material. *Trichoderma pseudokoningii* SIWT 51 produced no significant increases in any of the Sitka spruce material while *T. aureoviride* and *T. viride* SIWT 70 gave the largest number of significant increases.

		SITKA SPRUCE							
		Sapwood				Heartwood			
		Fresh		Dried		Fresh		Dried	
Organism	time (weeks)	Mean	Std	Mean	Std	Mean	Std	Mean	Std
T. aureoviride SIWT.1	5	11.91	6.91	6.58	14.71	-10.96	10.47	-1.46	13.52
	10	19.55	21.34	34.21	31.81	30.76	12.04	-8.94	10.88
T. polysporum IMI 206039	5	-8.38	13.66	13.99	13.26	6.29	8.61	-22.45	6.79
	10	3.43	27.23	16.96	25.91	25.97	12.72	-5.53	8.58
T. pseudokoningii SIWT 51	5	-6.38	17.37	7.22	13.97	4.10	10.71	-4.11	2.40
	10	1.67	24.21	11.50	3.97	14.21	11.89	-13.79	10.56
T. pseudokoningii SIWT 64	5	-0.25	12.91	20.53	14.08	0.37	16.82	-1.49	15.86
	10	31.38	9.50	13.75	5.45	12.14	12.34	-16.51	7.75
T. viride SIWT 70	5	14.22	10.80	1.20	6.39	-1.57	7.71	2.69	5.06
	10	92.25	71.62	31.43	34.39	19.31	16.10	-7.90	6.41
T. viride SIWT 100	5	-18.35	11.62	6.56	7.80	-6.70	16.93	-7.91	6.17
	10	38.17	23.89	44.01	30.47	27.02	10.94	-13.01	7.65

Table 4.6. Mean % increase in % uptake of decalin by fresh and dried Sitka spruce wood blocks after incubation with selected *Trichoderma* isolates for 5 or 10 weeks.

When analysed as mean % increase in decalin uptake (%) the results show more significant increases in permeability (Table 4.4) than with the earlier analysis (Table 4.3). This reflects the fact that the earlier analysis was purely based on the increase in decalin uptake. As the permeability of blocks was improved the uptake of decalin would be increased. Large increases in uptake indicated that the increase in permeability of the blocks had significantly increased. A set of blocks with a low initial permeability e.g. Dried Sitka spruce heartwood may be improved by the action of *Trichoderma* isolates but because of the low starting permeability any increase is not significant unless expressed as a % of the increase in uptake (%) of decalin. This will remove the actual value of the decalin uptake from the analysis and investigate purely the increase in uptake as a function of the level of permeability.

Comparison of table 4.4 with the same analysis of results from the original experiment (table 4.1) indicates that there are generally more significant increases in permeability recorded in the repeat experiment. An apparent anomaly in the comparison of the results of the repeat experiment with the original is that no significant improvement in permeability has been recorded in the dried Sitka spruce heartwood despite the fact that all organisms improved the permeability of this material in the earlier experiment. This can be explained due to the low initial moisture content slowing down colonisation of this material in the second experiment (table 4.7)

Fresh/Dried	Wood type	Initial moisture (%) (Dry Wt basis)	St. Dev	Final moisture (%) (Dry Wt basis)	St. Dev
Fresh	Scots pine sapwood	141	25	159	18
	Scots pine heartwood	33	9	169	30
	Sitka spruce sapwood	230	40	188	25
	Sitka spruce heartwood	81	26	165	24
Dried	Scots pine sapwood	10	1.4	121	47
	Scots pine heartwood	23	8	79	22
	Sitka spruce sapwood	20	3	127	32
	Sitka spruce heartwood	9	9	114	31

Table 4.7 Mean moisture contents of wood blocks before and after incubation with *Trichoderma* isolates.

Although isolate *Trichoderma viride* SIWT 100 (which was included as a negative control) produced a number of significant improvements in permeability (table 4.4) it is noticeable that when gross uptake of decalin was considered (table 4.3) it is found to produce significant improvements only in dried Scots pine sapwood.

4.2.4 Discussion

Trichoderma are pioneer soil dwelling organisms which when disturbances occur colonise and grow rapidly into new sites sometimes to the exclusion of other organisms (Hulme and Shields, 1972 (b); Schoeman *et al*, 1994). With *Trichoderma* being such a large *genus* there is variation in the abilities of different isolates and species to grow and compete on different substrates and isolates will respond differently to different growth

conditions. Schoeman *et al* (1994) reported that an isolate of *Trichoderma harzianum* selected for its biological control properties showed no evidence of permeability enhancement of Scots pine wood block samples. The isolates used in this experiment also showed much variation in their ability to improve the permeability of the different wood types. The isolates tested in this experiment to increase permeability of wood blocks were selected (chapter 2) on their ability to produce cellulase, pectinase and amylase. It should be noted however that the ability to produce these enzymes in pure culture in artificial medium does not guarantee that the organisms will be able to produce them *in situ* in wood or that the enzymes if produced will be active under such conditions. This may indeed explain why isolates of *Trichoderma pseudokoningii* SIWT 51 and 64 did not consistently produce significant increases in permeability in some of the wood material and conversely may explain the degree of increases shown by the negative control *Trichoderma viride* SIWT 100.

Generally the organisms showed the greatest increases in permeability enhancement when incubated with Scots pine sapwood. Scots pine sapwood is normally used as the bench mark for standard biological degradation tests as most organisms are capable of growing through this material. Comparing the pine sapwood to the heartwood material, sapwood will be more readily colonised by the organisms because of the presence of sap sugars and the likelihood of fewer extractives in this region.

The results of the GLM model depicted in Table 4.5. indicate that more significant increases in permeability were observed in the dried pine sapwood material than of fresh sapwood material. These increases may be linked to the dried material having fewer inhibitors present and being physiologically dead when exposed to the *Trichoderma*. This would make the timber more susceptible to colonisation by the isolates. The dried material was also sterilised prior to incubation with the *Trichoderma* isolates which would eliminate competition from other organisms during the experiment. The fresh material was not sterilised and may have had resident organisms that would have to be overcome before the isolates would grow through the timber. Fresh timber can remain

physiologically active for a considerable period after felling and cutting. This fact and competition from residual organisms may explain why only a few isolates were able to improve significantly the permeability of the wood blocks after 5 weeks while most showed significant increases after 10 weeks.

Pine heartwood is considered to be moderately durable (Hue,1992) and growth of organisms through the timber can be inhibited by the presence of extractives and extraneous compounds that are deposited during differentiation of the timber in this region. Carey *et al* (1984) showed that after leaching, the decay resistance of Scots pine heartwood could be significantly reduced, indicating that some of the chemicals giving decay resistance to the timber are water soluble. The loss of extractives from air dried Scots pine heartwood may be responsible for the larger number of isolates that showed improvement in the permeability of this material compared with the fresh heartwood material.

Despite the presence of extractives in the heartwood of fresh Scots pine the isolates were able to grow in the presence of these compounds and improve the permeability of the timber significantly.

The abilities of the isolates to improve the permeability of Sitka spruce were generally poorer than with the pine material. Spruce is a less durable material that does not rely on the same chemical methods for protection from invading organisms. Scheffer and Cowling (1966) indicated however that the C:N ratio could limit the growth of organisms through woody plant tissues. In most wood species this ratio is 350-500: 1 but in Sitka spruce heartwood this ratio is as high as 1250:1. For most organisms a high C:N ratio will provide a growth medium that is deficient in nitrogen and will not support growth. The lack of nitrogen in the heartwood of spruce may reduce the ability of the *Trichoderma* isolates to produce the necessary degradative enzymes and be responsible for the lack of permeability improvement observed with the dried heartwood material (Scheffer and Cowling, 1966). The fresh sapwood material may also have some sap sugars present which would permit some growth through the wood blocks.

Fresh Sitka spruce material also showed similar properties in the timing of improvements in permeability to that of Scots pine. All of the improvements in permeability were seen after 10 weeks incubation with the selected *Trichoderma* isolates. This may be due to the competition of *Trichoderma* with other organisms or may be due to the higher moisture slowing the establishment of the fungus and hence no improvements were seen during the first 5 weeks of incubation. Generally the *Trichoderma* isolates did not improve the permeability of the spruce material as easily as the pine material. Blocks incubated with the *Trichoderma* isolates were visually better colonised in the repeated experiment. The lack of significant permeability improvement in dried Sitka spruce heartwood in the repeated experiment may be due to the following:

i) The decalin method may not be sensitive enough to detect improvements in the permeability in timber with low inherent natural permeability; however this is unlikely as significant improvements were seen in the pine heartwood which has a permeability similar to the spruce material.

ii) The growth and sporulation of the organism on or through the timber may have reduced or masked permeability changes by blocking cut surfaces and preventing decalin uptake. The latter factor may have greater implications as the blocks were cleaned as much as possible on removal from the incubation jars but not all of the spores etc. could be removed from the blocks giving rise to the possibility of blocked pits on the surface of the wood blocks.

Figures 4.9-4.16 show the differences in mean % uptake of decalin by the different treatments and wood types. Most wood types show positive increases in permeability but due to variation within the samples these increases in permeability are not significant. With the less permeable material these increases in permeability are comparatively small as the uptake of decalin is lower than for more permeable material e.g. pine sapwood. When these increases in permeability are represented as % increases in uptake (%) of decalin, material with low permeabilities can show larger increases than other material. e.g. Pine sapwood control with a decalin uptake of 10% (based on a dry wt basis of the

block) and a *Trichoderma* treated uptake of 15 % would gave a difference of 5 % but the treated block will have an uptake that is 50 % higher than the control. Hence material with low permeabilities can have significant increases in permeability despite having small real increases in uptake.

From table 4.7 it can be seen that in this experiment the moisture content of the timber was not significant in preventing the colonisation as in the previous experiment (table 4.1) and hence the results are more representative of the organisms' true abilities to improve the permeability of the different timber types.

From the results of this experiment it can be seen that two isolates have performed consistently on most of the wood types. *Trichoderma aureoviride* SIWT1 and *Trichoderma viride* SIWT 70 were therefore selected for use in further trials on roundwood logs. Although there were doubts concerning the validity of the results from the first experiment described in section 4.1 the two isolates selected on the basis of the repeated experiment also gave the highest numbers of significant increases in permeability in the original experiment.

Chapter 5. Air Permeability Determinations and the Effect of *Trichoderma* on the Permeability of Wood Samples.

5.1 Introduction.

The physical basis of water conduction in plants and governing forces were first set out by and Dixon and Jolly (1894) and their theories still provide the most widely accepted explanation of sap flow in plants. Water movement, which largely occurs in the non-living elements of the xylem is assumed to be a response to purely physical forces rather than a vital process. If this is so, the permeability (conductivity) of wood should be a clear physical property of wood amenable to Darcy's law which describes viscous flow through porous media (Gregory, 1977).

Wood permeability has been measured using either fluid uptake or flow through wood samples (Comstock, 1967). Earlier work (Chapters 3 & 4) used a liquid uptake method. This uptake method relies on blocks cut from wood samples and is basically a destructive method of measuring wood permeability. If the effects of *Trichoderma* isolates on the permeability of roundwood logs were to be determined then a less destructive method for sampling and measuring wood permeability would have to be developed. Since the decalin method was not sensitive enough to show differences in drying regimes (Chapter 3) it was necessary to develop a method for measuring air permeability in round wood samples.

Measuring wood permeabilities using gas flow through wood samples has been used by several researchers (Bailey, 1964; Comstock, 1965; Johnstone, 1967; Perng, 1980 a,b and c; Bolton, 1988). Permeability, the fluid conductivity of a material, is a measure of the ease with which fluids may be made to pass through the material under a pressure gradient. Permeability though characterised by Poiscuille's law for flow through pipes and tubes, was first defined in terms of measurable quantity for porous media by Darcy in 1856 in the form, which is now called Darcy's law.

The equation to be used to determine wood permeability is as follows:

$$Kg = \frac{q\ L\ P\ \eta}{A\ P\ \Delta P}$$

Where Kg = the specific permeability of the sample material.

q = Flow rate through specimen (cm^3/sec).

L = Length of specimen (cm).

P = Pressure at which Flow rate is measured (atm).

A = cross sectional area of specimen (cm^2).

ΔP = pressure difference across specimen (atm).

$\bar{P}$ = average pressure in specimen (atm).

η = viscosity of fluid (centipoise).

A derivation of this equation is documented by Kumar (1981) and although Darcy formulated this equation for sand, some studies have proved its applicability to wood with a variety of fluids (Comstock, 1965; Johnson, 1967; Kumar, 1981). If Darcy's law was to be followed to evaluate the permeability of wood samples then a method to measure all of the required parameters is needed.

The aim of this chapter was to design and use an air permeability method for measuring wood permeability.

5.1.2 Methods

The measurements of the specimens' dimensions was done using callipers accurate to the nearest 0.1mm. The other components posed greater problems, initial work was done on setting up a pressurised system that would pass air through wood blocks. Trials showed that an air compressor would not pass measurable amounts of air through Sitka spruce blocks. The compressor was replaced with a cylinder of compressed air and with this reservoir air was seen to pass through spruce wood blocks. The problem of sample

preparation was overcome by using a plug cutter with an internal diameter of 12.5 mm to cut cores from wood samples. Use of the plug cutter eliminated the need for cuboidal wood blocks and gave a method for sampling round wood logs without destroying the whole log. However there were still problems with mounting the plugs in the apparatus for permeability analysis. Further trials were conducted using a sample holder in which the cores were mounted against a spring and sealed with an O- ring to prevent air slipping around the side of the sample. As the air pressure was increased it was found that sample cores were pushed against the mounting spring breaking the seal around the O-ring and allowing air to pass around the core thereby giving a false measurement of permeability. Failure to provide a complete seal of the cores against the O-ring also gave some problems with reproducibility when cores were re-measured.

The mounting of the cores was then redesigned and cores were placed in a specimen holder that consisted of thick walled rubber tubing of 11.5mm internal diameter. The edges of samples were sealed in silicone grease and then clamped in place to prevent air leakage around the edges of the sample.

The flow rate was measured by using an air flow meter supplied for the calibration of a Gas- Chromatography machine. This was basically a soap bubble that was pushed by the air passing through the sample and hence a time could be measured for a given volume of air to be passed through the sample.

The pressures in the experiment were measured with a hand held manometer. A digital monitor was used for the pressure measurements since using other materials would have necessitated manometers filled with large amounts of either mercury or water.
The air was passed through desiccant ($Mg(ClO_4)_2$) prior to being passed through the sample as contaminating water vapour in the air would have introduced further errors into the experiments.

The apparatus was set up as in Figure 5.1.

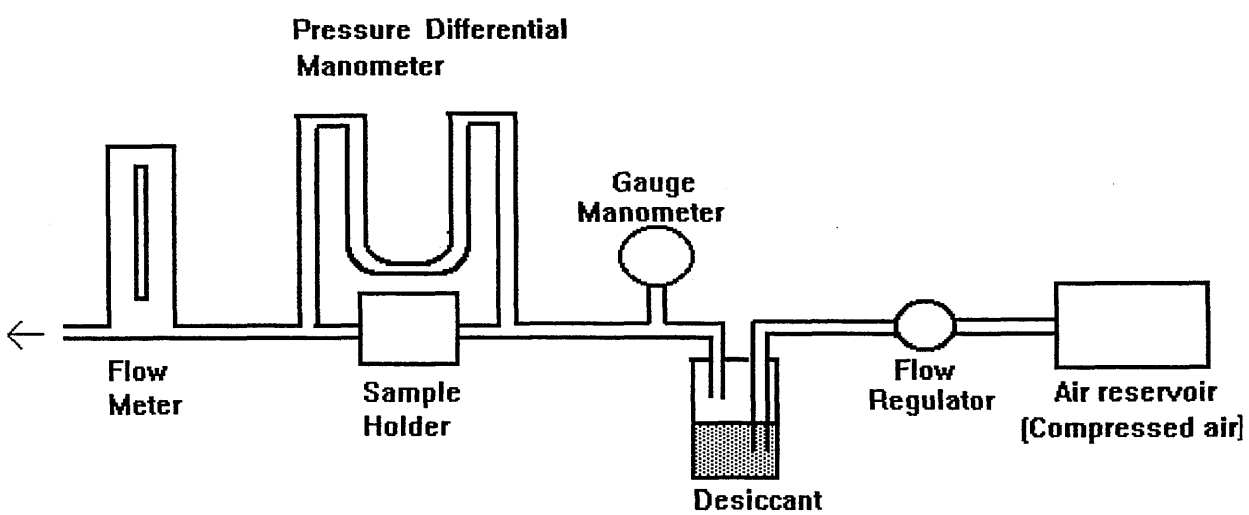


Figure 5.1 Diagrammatic representation of apparatus used to measure air permeability.

The flow time of a set volume of air was measured, the pressure difference and running pressure noted and the length and diameter of the samples measured. From these measurements the permeability of the cores could be determined using the equation given

in section 5.1.2. Trial measurements were also carried out on impermeable specimens sealed in wax to ensure that this technique excluded measurable leaks.

Initial trials on wood permeability of different wood types and orientations were undertaken. Cores (15mm x 12.5mm diameter) were cut from fresh roundwood pine and spruce logs to give 15 samples with which to measure the permeability in each of the longitudinal, radial and tangential directions.

5.1.3 Results

Wood species	Wood type	Permeability direction	Permeability (Darcys x 10^4)
Scots pine	Sapwood	Longitudinal	13250
		Radial	440
		Tangential	30
	Heartwood	Longitudinal	1198
		Radial	20
Sitka spruce	Sapwood	Longitudinal	2067
		Radial	20
		Tangential	3
	Heartwood	Longitudinal	30
		Radial	2

Table 5.1. Mean air permeabilities of samples of Scots pine and Sitka spruce cored in different directions. N.B. Due to the small diameter of sample logs insufficient heartwood was available to obtain tangential heartwood sections.

The results shown in table 5.1 show that the permeability of the pine sapwood and heartwood samples is greater than for the similar spruce material. For both timber species the permeability of the heartwood and sapwood is greatest in the longitudinal direction. The permeability in the longitudinal direction is several times greater (here up to 30 times) than in either the radial or tangential directions. These permeability results show similar trends to those seen for decalin uptake in different directions (Chapter3 Section 3.6) and between the two species. The results shown in table 5.1 are for relatively small sample sizes (15 per wood type and direction) but do show the general trends in the different flow directions.

5.1.3 Discussion

These results show that the permeabilities observed from fresh Scots pine and Sitka spruce samples are similar to those reported by Comstock (1970). In both species the permeability in the longitudinal direction was greatest; the radial direction was lower and the tangential direction the lowest. When these results were compared to the decalin uptakes similar patterns were observed. The permeabilities of samples from heartwood regions of both species were lower than those observed in the sapwood regions of the same species.

Differences in permeability in these regions are again due to the structural differences that exist between the sapwood and heartwood regions. Generally in heartwood material most bordered pits are aspirated and encrusted with extraneous material (Hillis, 1987). Sapwood by definition carries sap throughout the tree and bordered pits in this region will normally be open. On cutting or damage an air interface is introduced to the fibre and this causes aspiration of the pit membrane reducing permeability. In the sapwood these aspirated pits can still allow air to pass through as small apertures in the membrane allow fluids under pressure to pass through. This gives the sapwood a higher permeability than the heartwood as these apertures are encrusted in the heartwood material. Also on drying

more of the pits in the sapwood can remain open giving rise to a higher permeability (Wiedenbeck *et al*, 1990).

Air permeability will be affected by the number and state of bordered pit membranes that have to be crossed over a given distance within the timber. Differences in air permeability in different flow directions through the wood are therefore due to structural differences in these directions (figure 5.2).

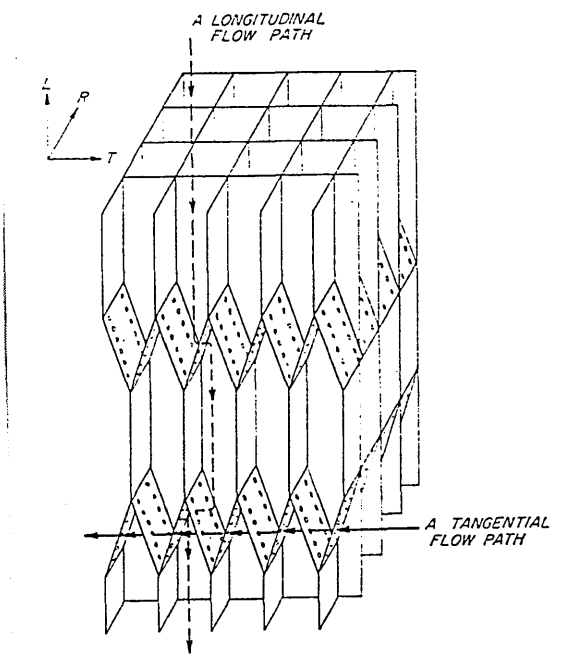


Figure 5.2 Diagram of model for fluid flow through wood.(after Comstock 1970)

In the longitudinal direction fewer bordered pits must be negotiated as tracheids run in this direction and hence the permeability in this direction is greater. In the tangential flow direction more bordered pits must be crossed than in any other direction and hence the permeability in this direction is lower. Radial flow is reliant on the same bordered pits as in the tangential flow but will also traverse medullary rays and resin canals running in this direction which will make the wood more permeable than in the tangential direction.

The approximate volumetric composition of the wood of *Pinus strobus* is:

Longitudinal tracheids 93%

Longitudinal resin canals 1%

Wood rays 6%

This is a typical softwood composition, ray volumes may range from 3.4 to 11.7% for softwoods with an average of 7%, contrasted with an average of 17% for hardwoods (Siau, 1984). Since the rays and resin canals form but a small fraction of the volume, their contributions to the overall flow in the living tree may be of secondary importance. There is some evidence that rays are important for timber preservation and that rate of uptakes in the radial direction correlates well with the known treatability of timber species (McQuire, 1970).

5.2 VARIATION IN INTER- AND INTRA- LOG PERMEABILITY.

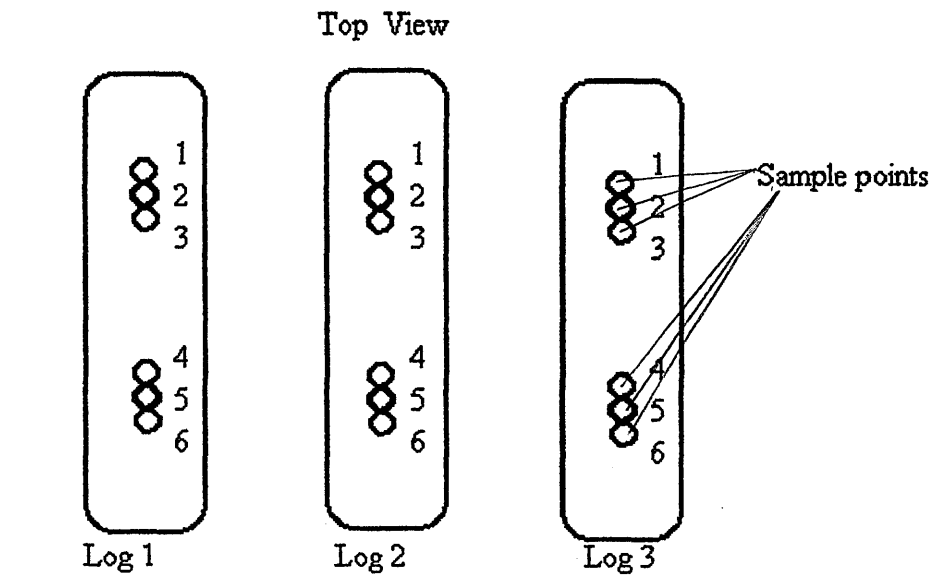
5.2.1 Introduction

The permeability of timber is seen to vary between different wood species and within the same species (Comstock, 1970). Any factor that affects the growth and development of the tree in the forest stand will affect the subsequent structure and permeability of the timber. This will include a combination of the weather, growth environment and physiological structure of the timber. This will result in the permeabilities of trees within a stand being completely different to each other as environmental factors will affect each tree differently. Even within the tree itself different stresses will affect the tree as it grows and hence wood will be laid down under different conditions and will give rise to different permeabilities. This variation in growth conditions will cause variation in the permeability and if *Trichoderma* spp. are to be used to improve the permeability of the timber species it is necessary to investigate the amount of variation that may occur within and between logs

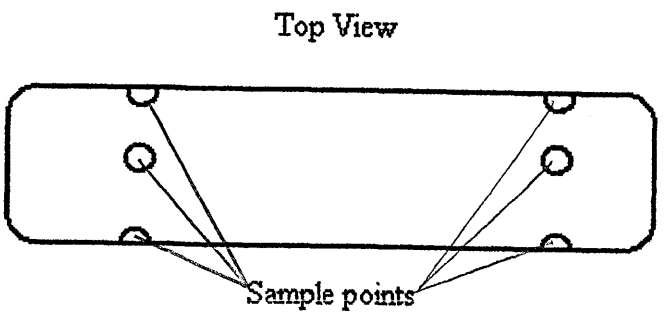
of the same species, hence the aim of the following experiment is to investigate the inter- and intra- log variation of Scots pine and Sitka spruce logs.

5.2.2. Methods

Samples were prepared from fresh Scots pine and Sitka spruce logs using a plug cutter and pillar drill. Cores were cut in a radial direction from various sites on different logs and a diagram of the sampling regime can be seen in figure 5.3. Cores were cut in groups of 3 at each sample site. The cores were then matched and cut into 1cm sections down the length of the core. The sections were numbered, weighed and dried down at 103^{o}C to constant weight. Sections were then re-weighed and the moisture content calculated, this process allowed the differentiation of cores into those that contained either sapwood or heartwood portions and also those cores at the boundary containing both heartwood and sapwood. The permeability of the sections that contained only sapwood or heartwood were determined and the results for similarly treated samples in adjacent cores compared.
In order to establish whether permeability changed significantly at radial sites around the logs, core samples were also removed from 3 equally spaced points around the circumference of 2 Scots pine and 2 Sitka spruce logs. Cores could not be cut from adjacent sites in this direction, as core samples would be incomplete because of the curvature of the logs.



Samples removed from along the length of the top surface of the logs.



Cores sampled in a radial direction around the log

Figure 5.3 Diagram of sampling used to determine inter and intra log variation.

For each group of matched cores, samples were cut from sites as close as possible in a longitudinal direction. Cores removed around the logs would be subject to much greater variations in permeability as these cores would be cut in the tangential direction and open to more variation in permeability.

5.2.3. Results

Permeability results from cores removed from adjacent sites along freshly felled Scots pine and Sitka spruce logs can be seen in tables 5.2 and 5.3 respectively.

Log 1				Log2	
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sap	220		Sap	323
1.2	Sap	395		Sap	69
1.4	Heart	64		Heart	2
1.5	Heart	42		Heart	2
2.1	Sap	155		Sap	444
2.2	Sap	301		Sap	51
2.4	Heart	17		Heart	4
2.5	Heart	11		Heart	2
3.1	Sap	259		Sap	350
3.2	Sap	324		Sap	42
3.4	Heart	11		Heart	5
3.5	Heart	12		Heart	1
4.1	Sap	442		Sap	105
4.2	Sap	497		Sap	129
4.4	Heart	69		Heart	1.5
4.5	Heart	43		Heart	2.7
5.1	Sap	361		Sap	123
5.2	Sap	446		Sap	128
5.4	Heart	6		Heart	4
5.5	Heart	18		Heart	7
6.1	Sap	346		Sap	94
6.2	Sap	402		Sap	105
6.4	Heart	15		Heart	7
6.5	Heart	21		Heart	3

Table 5.2 Permeability of freshly felled Scots pine samples remove from adjacent sites along the log .

Log 3				Log 4	
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sap	109		Sap	170
1.2	Sap	55		Sap	170
1.4	Heart	1		Heart	3
2.1	Sap	72		Sap	166
2.2	Sap	70		Sap	146
2.4	Heart	3		Heart	5
3.1	Sap	85		Sap	103
3.2	Sap	64		Sap	169
3.4	Heart	0.75		Heart	7
4.1	Sap	181		Sap	200
4.2	Sap	155		Sap	146
4.4	Heart	12		Heart	0.4
5.1	Sap	153		Sap	151
5.2	Sap	124		Sap	108
5.4	Heart	10		Heart	4
6.1	Sap	103		Sap	141
6.2	Sap	113		Sap	94
6.4	Heart	4		Heart	5

Table 5.2 continued.

Log 5			Log 6		
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sap	48		Sap	100
1.2	Sap	34		Sap	64
1.4	Heart	0.5		Heart	0.5
2.1	Sap	31		Sap	138
2.2	Sap	30		Sap	108
2.4	Heart	0.3		Heart	2
3.1	Sap	57		Sap	105
3.2	Sap	37		Sap	80
3.4	Heart	2		Heart	0.9
4.1	Sap	130		Sap	119
4.2	Sap	97		Sap	59
4.4	Heart	5		Heart	0.9
5.1	Sap	108		Sap	82
5.2	Sap	106		Sap	47
5.4	Heart	3		Heart	1.5
6.1	Sap	122		Sap	95
6.2	Sap	84		Sap	64
6.4	Heart	9		Heart	0.4

Table 5.2 continued Permeabilities (Darcys $x10^4$) of core samples removed from adjacent sites in freshly felled Scots pine logs. (Sapwood and heartwood differentiation is based on moisture content). NB. sample depth 3 is not reported as this sample was comprised of both heartwood and sapwood material.

It is clear from results in table 5.2 that there is large inter log variability in permeability i.e. comparing log 1 and 5 and indeed permeabilities taken from different locations in the same log vary significantly e.g. the sapwood of log 1 is double the value found at the other sample location approximately 50cm away. Despite these intra and inter variations the results in table 5.2 show that samples removed from similar depths in adjacent cores have similar permeabilities. The results also show clearly measurable differences in the permeability of the sapwood and heartwood samples in Scots pine.

Log 1				Log2	
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sap	14.1		Sap	28.5
1.2	Sap	6.9		Sap	2.3
1.4	Heart	0.9		Heart	1.4
2.1	Sap	23.7		Sap	42.6
2.2	Sap	3.8		Sap	6.7
2.4	Heart	0.2		Heart	1.5
3.1	Sap	21.0		Sap	29.9
3.2	Sap	4.8		Sap	4.5
3.4	Heart	0.2		Heart	0.1
4.1	Sap	22.7		Sap	14.8
4.2	Sap	6.6		Sap	7.8
4.4	Heart	2.8		Heart	3.8
5.1	Sap	12.7		Sap	33.4
5.2	Sap	2.9		Sap	19.2
5.4	Heart	1.1		Heart	1.1
6.1	Sap	12.5		Sap	22.5
6.2	Sap	3.6		Sap	4.7
6.4	Heart	0.9		Heart	3.6

Table 5.3 Permeabilities (Darcys x 10^4) of freshly felled Sitka spruce samples remove from adjacent sites along the log .

Log 3			Log 4		
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sap	7.0		Sap	13.4
1.2	Sap	1.64		Sap	19.15
1.4	Heart	1.52		Heart	12.74
2.1	Sap	10.5		Sap	15.12
2.2	Sap	2.84		Sap	12.56
2.4	Heart	0.13		Heart	11.43
3.1	Sap	10.0		Sap	16.95
3.2	Sap	1.21		Sap	13.84
3.4	Heart	0.64		Heart	10.29
4.1	Sap	1.76		Sap	0.37
4.2	Sap	1.30		Sap	0.34
4.4	Heart	1.48		Heart	1.5
5.1	Sap	1.15		Sap	0.59
5.2	Sap	1.43		Sap	0.31
5.4	Heart	3.4		Heart	0.64
6.1	Sap	5.82		Sap	0.54
6.2	Sap	2.0		Sap	1.0
6.4	Heart	2.5		Heart	0.8

Table 5.3 Continued.

Log 5			Log 6		
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sap	0.95		Sap	0.2
1.2	Sap	0.88		Sap	0.05
1.4	Heart	4.05		Heart	1.89
2.1	Sap	0.70		Sap	0.33
2.2	Sap	1.64		Sap	0.15
2.4	Heart	6.07		Heart	2.31
3.1	Sap	0.34		Sap	1.16
3.2	Sap	0.66		Sap	0.76
3.4	Heart	5.39		Heart	0.86
4.1	Sap	5.31		Sap	5.77
4.2	Sap	0.69		Sap	0.59
4.4	Heart	3.32		Heart	12.10
5.1	Sap	5.65		Sap	1.56
5.2	Sap	0.79		Sap	0.37
5.4	Heart	3.08		Heart	9.95
6.1	Sap	5.3		Sap	6.02
6.2	Sap	2.42		Sap	1.9
6.4	Heart	1.54		Heart	8.55

Table 5.3 Permeabilities (Darcys x10^4) of core samples removed from adjacent sites in freshly felled Sitka spruce logs. (Sapwood and heartwood differentiation is based on moisture content). NB. sample depth 3 is not reported as this sample was comprised of both heartwood and sapwood material.

As in Scots pine the results in table 5.3 show that large inter and intra log variation exists in the permeability of the Sitka spruce material. Again however despite these differences samples removed from adjacent points again show similar values for permeability of the cores. The results of cores removed around the logs can be seen in tables 5.4 and 5.5 for Scots pine and Sitka spruce respectively.

Log 1				Log 2	
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sapwood	490		Sapwood	578
1.2	Sapwood	640		Sapwood	420
1.4	Heartwood	21		Heartwood	15
2.1	Sapwood	155		Sapwood	259
2.2	Sapwood	335		Sapwood	324
2.4	Heartwood	12		Heartwood	12
3.1	Sapwood	442		Sapwood	422
3.2	Sapwood	397		Sapwood	490
3.4	Heartwood	126		Heartwood	90

Table 5.4 Permeabilities (Darcys x 10^4) of Scots pine radial cores removed from sites around the logs.

Log 1				Log 2	
Core No	Sap/ Heartwood	Permeability		Sap/ Heartwood	Permeability
1.1	Sapwood	12		Sapwood	25
1.2	Sapwood	9		Sapwood	44
1.4	Heartwood	5		Heartwood	2
2.1	Sapwood	134		Sapwood	117
2.2	Sapwood	108		Sapwood	34
2.4	Heartwood	27		Heartwood	6
3.1	Sapwood	91		Sapwood	22
3.2	Sapwood	31		Sapwood	7
3.4	Heartwood	5		Heartwood	1

Table 5.5 Permeabilities (Darcys x 10^4) of Sitka spruce radial cores removed from sites around the logs.

The figures in tables 5.4 and 5.5 show that the permeabilities around the logs at a particular height are not as closely related as those observed from adjacent sites along the log (Tables 5.2 and 5.3). These will be related to the different stresses imposed on the trees during growth, i.e. different compressions on the timber will be experienced because

of factors like prevailing wind direction and strength, which will affect the differentiation and growth of new cells. Obviously the growing tree will require stability during growth hence cells will be laid down in differing amounts during different growth periods and this will also affect the permeability of the timber.

5.2.4 Discussion.

The positions of cores removed from the logs will have an effect on the permeability of the resulting samples. Table 5.1 showed the effects of different orientations of the cores on the permeability, tables 5.4 and 5.5 show the differences between sample sites on the same logs and between different logs. With cores removed from adjacent sites the permeability appears to be roughly similar for equivalent positions in the cores. Obviously the permeabilities of these sample cores will never be the same because of the structural differences that exist at the sampling points. By removing cores from adjacent sites it was hoped to remove as much variation as possible as the timber in these regions would be subjected to similar stresses when growing and may show similar amounts of the different wood cell types during growth. By removing as much of the variation in growth it was hoped that the cores removed from adjacent sites would show similar permeabilities and could thereby be used to provide appropriate reference samples for logs which would subsequently be inoculated with *Trichoderma* (see chapter 6).

Results for both pine and spruce show roughly similar permeabilities in cores removed from adjacent sites but differing permeabilities when compared with other sites in the logs and with other logs. This result highlights the different pressures that will be exhibited during tree growth and hence mirrors structural differences within the timber. The permeabilities of late wood and early wood have been reported to differ with late wood being more permeable because of the resistance of late wood to aspiration (Bolton and Petty, 1978). With varying growth conditions the proportions of early wood/ late wood which are laid down will differ and hence differences in permeability will result. Differences in permeability may also be related to tree age. Growth rings generally become smaller as the growing tree grows older. This may alter the amounts of early

wood/ late wood over a given distance within the tree thereby influencing the permeability. The width of growth rings will also vary with height, as the higher up the tree the younger the timber that has been laid down, and hence this will differ in permeability to that of samples removed from lower down the tree (Hillis, 1987).

5.3 EFFECT OF *TRICHODERMA* ISOLATES ON THE PERMEABILITY OF LONGITUDINAL CORES FROM FRESH SCOTS PINE AND SITKA SPRUCE SAMPLES.

5.3.1 Introduction.

The permeability of the radial cores (Section 5.2.) shows that there is variation within and between sample logs. As previously described the permeability of cores removed from adjacent sites appear to be generally similar but if this method is to be used to determine if selected *Trichoderma* isolates are improving the permeability of treated timber, it is necessary to know to what extent the *Trichoderma* isolate can improve permeability of treated samples. The aim of this section was to undertake a preliminary investigation of the effect of selected *Trichoderma* isolates on the permeability of samples of Scots pine and Sitka spruce in a longitudinal direction.

5.3.2. Methods.

Cores (1.25cm diameter) were cut using a plug cutter from discs of fresh Scots pine and Sitka spruce sapwood and heartwood to give samples that could be used to measure the permeability in a longitudinal direction. This orientation was selected as it would give best matched samples since variation down longitudinal cores is less than in any other direction (Johnson, 1967). The cores were sectioned into 1.5 cm lengths (see figure 5.4) to provide matched pairs from adjacent sites along the core length. The samples were then weighed and one of each matched pair exposed to the selected *Trichoderma* isolates (*Trichoderma aureoviride* SIWT 1 or *Trichoderma viride* SIWT 70). Twelve replicates were used for each treatment. The unexposed control cores were then dried down at

103°C and stored over desiccant (Silica) until the completion of the experiment. Cores were incubated with the selected isolates at 22 or 25°C for 4 weeks before being weighed and dried down at 103°C. The air permeability of the paired wood samples was then measured as described in Section 5.1.2 . The results of the air permeability determinations of the treated were then compared with the matched core samples. The change in permeability (%) was calculated for each pair with the difference in permeability being expressed as a percentage of the untreated core sample.

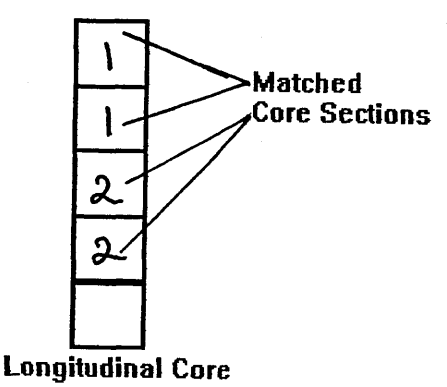


Figure 5.4 Sectioning of longitudinal cores into matched pairs.

5.3.3 Results.

The mean change in the longitudinal permeability (%) of cores of Scots pine and Sitka spruce heartwood and sapwood compared with matched controls after exposure to *Trichoderma aureoviride* SIWT 1 and *Trichoderma viride* SIWT 70 are shown in table 5.7

	Trichoderma aureoviride SIWT1		*Trichoderma viride* SIWT 70	
Wood type	Mean % Increase	Standard Deviation	Mean % Increase	Standard Deviation
Pine sapwood	164	130	193	157
Pine heartwood	59	41	29	18
Spruce sapwood	179	337	-5	377
Spruce heartwood	3	59	-28	29

Table 5.7 Mean increase in longitudinal permeability (%) produced by *Trichoderma aureoviride* SIWT1 and *Trichoderma viride* SIWT 70.

The results in Table 5.7 show that for pine sections there was a positive increase in permeability after exposure to the selected *Trichoderma* isolates. The spruce material showed lower increases in permeability and in fact showed some decreases in permeability. Since these isolates had been previously shown to improve the permeability of wood samples cut from fresh pine and spruce material using decalin it appears highly unlikely that these isolates should now reduce the permeability of the wood samples. One explanation however may be that the reduced permeability is due to mechanical blocking of pits by *Trichoderma* spores in these samples. To determine whether this reduction was caused by blocking of the cut ends of individual tracheids, the radial faces of the matched pairs were sectioned using a sledge microtome and the permeability of the cores re-measured. The results of these determinations can be seen in Table 5.8.

Wood species	Wood type	% increase in permeability of *Trichoderma aureoviride* SIWT1 treated cores	Standard Deviation	% increase in permeability of *Trichoderma viride* SIWT 70 treated cores	Standard Deviation
Sitka spruce	Sapwood Heartwood	1254 30	115 10	84 19	30 6

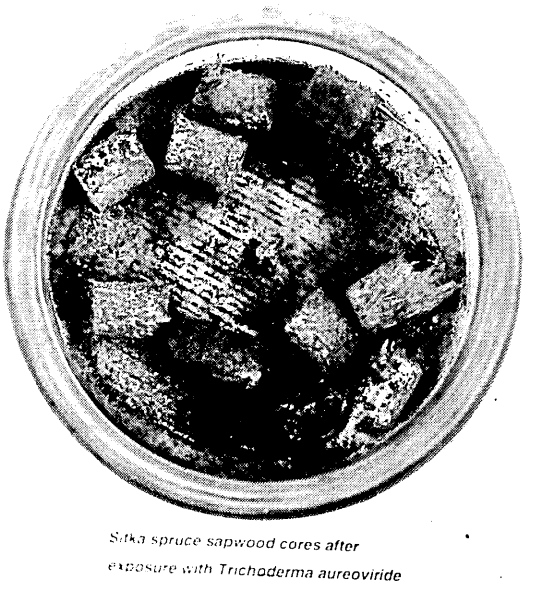
Table 5.8 Effect of selected *Trichoderma* isolates on the air permeability of longitudinal Sitka spruce cores after microtome removal of surface material.

The results in table 5.8 show that the spruce material suffered from the blocking of cut tracheids by spore material and that initial increases in permeability were quite low. Removal of the end sections of the cores by microtoming, however improved the permeability of the sapwood to the levels observed in Table 5.8. *Trichoderma aureoviride* appears to have increased the permeability of the sapwood by huge proportions compared with the other isolate. These results show positive increases in the permeability of the spruce sapwood and heartwood but actual values of the air permeability were still lower than those recorded in the more readily treatable pine sapwood material (e.g. after treatment with *Trichoderma* the permeability of Sitka spruce sapwood is approximately 0.006 Darcys compared with an untreated pine sapwood sample of 0.4 Darcys).

5.3.4. Discussion.

From this experiment it is evident that the permeabilities of the pine sapwood and heartwood were increased by the action of the *Trichoderma* isolates. The spruce material showed initial increases in permeability in some cores up to 500%, however a lower permeability was observed from other cores (a decrease of 70%) after exposure to the

Trichoderma isolates. Plate 5.1. shows the cores during incubation with the *Trichoderma* isolates and it can be clearly seen that core sections are covered with spores from the growing *Trichoderma* and these reductions in permeability were probably due to the blocking of pits and cut tracheids during the growth of the *Trichoderma* through and over the core sections.



Sitka spruce sapwood cores after exposure with Trichoderma aureoviride

Plate 5.1 . Jar containing core sections incubated with *Trichoderma aureoviride*.

From the results in table 5.8 it can be seen that the production of spores by the *Trichoderma* isolates effectively blocked the cut ends of the sections and significantly reduced the permeability of the cores. Sectioning the cut ends of the *Trichoderma* treated cores showed a significant increase in the permeability of the cores with *Trichoderma aureoviride* SIWT1 treated cores showing the highest increase in permeability. Despite these high increases in permeability in the spruce material the actual permeability of the treated spruce sapwood material is still below that seen with untreated pine sapwood and thus *Trichoderma* treatment may not improve the penetrability of the spruce material to preservative by enough to render this timber suitable for standard commercial creosote treatment.

5.4. COMPARISON OF LONGITUDINAL AIR PERMEABILITY AND DECALIN UPTAKE IN WOOD CORES

5.4.1. Introduction.

Since two methods have been used to determine the permeability of wood samples, the two different methods were investigated to show if any relationship existed between the two and whether increases in air permeability correlated well with increase in decalin uptake.

5.4.2. Methods

Cores (6.00cm x 1.25cm diameter) were cut from freshly felled Scots pine and Sitka spruce sapwood and heartwood, the cores were dried before being completely sealed by submerging them in hot wax. Samples (1.5cm x 1.25cm) were then cut from the cores with the end cores being discarded (figure 5.5). The permeability of the samples was then measured in the longitudinal direction to air and the same cores were subsequently weighed and immersed in decalin for 10 seconds after which they were blotted dry and re-weighed.

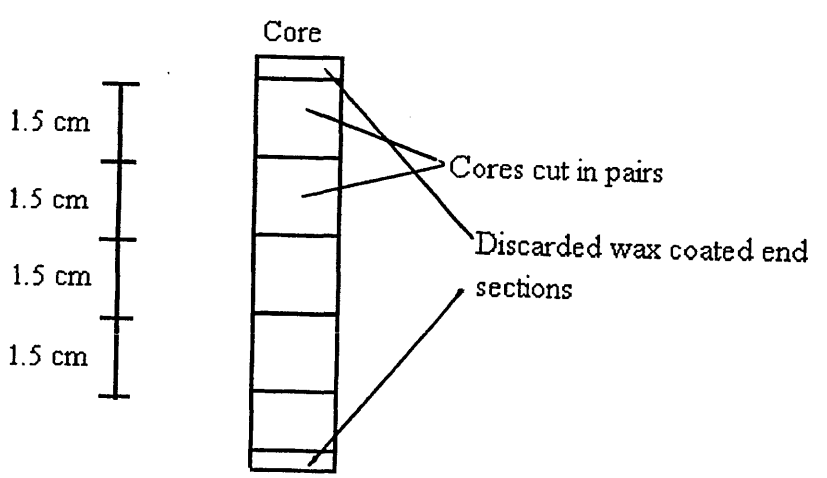


Figure 5.5 Sectioning of wax coated wood cores for air permeability and decalin uptake measurements.

5.4.3. Results

The air permeabilities and decalin uptakes were plotted against each other for all of the wood types and species, the results of this analysis can be seen in figure 5.6.

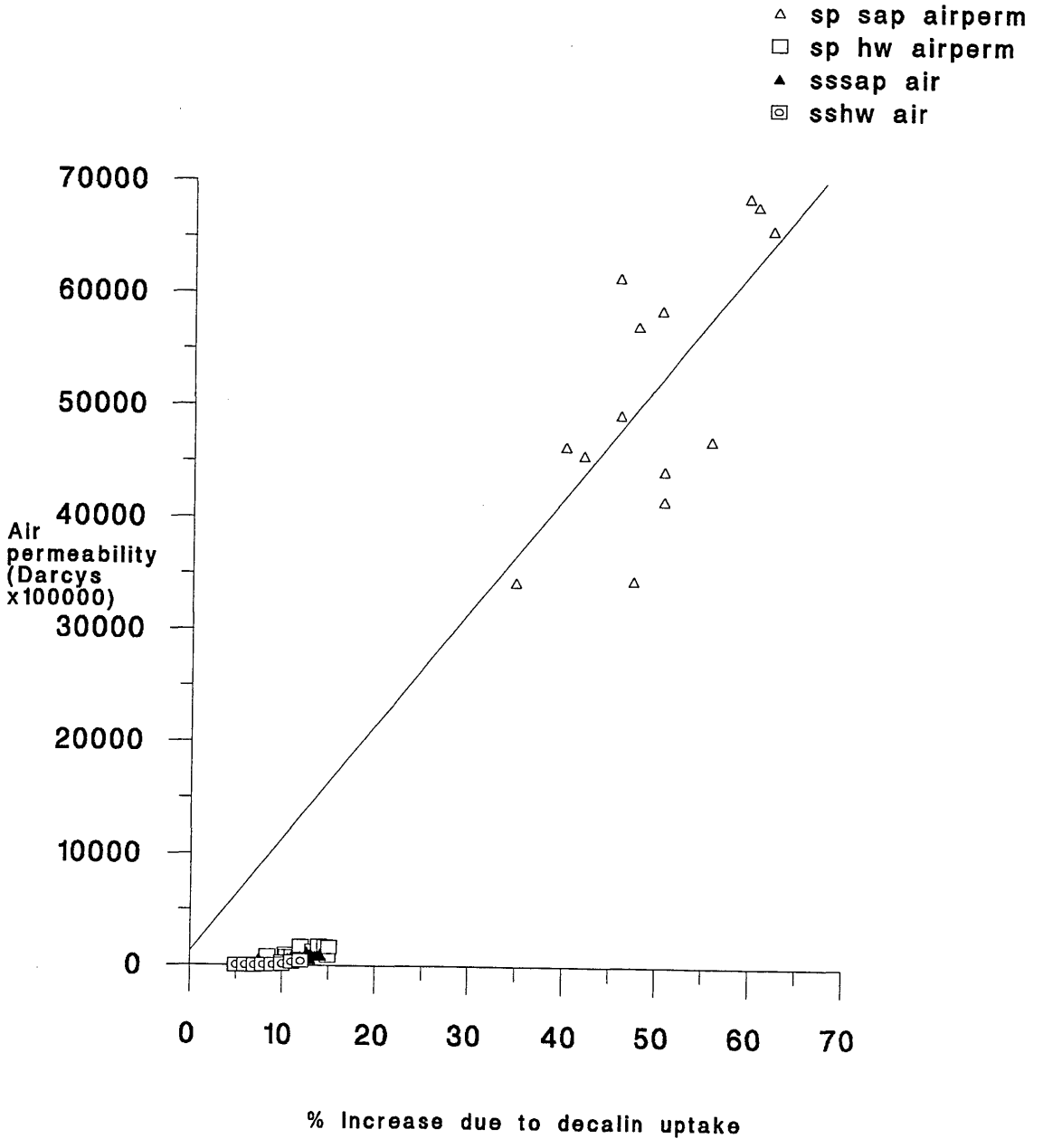


Figure 5.6 Comparison of air permeability and decalin uptake in Scots pine and Sitka spruce sapwood and heartwood. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

When the correlation co-efficient of this data was calculated it was found to be 0.97 thereby showing a good positive correlation between air permeability and decalin uptake after 10 seconds immersion.

5.4.4. Discussion

The results of the correlation analysis show a positive relationship between the air permeability and decalin uptake in a longitudinal direction with samples of Scots pine and Sitka spruce. This implies that any increases in air permeability caused by any biological pretreatment would also result in increases of decalin uptake and therefore may also be related to increased preservative uptake. Cooper *et al* (1974) also reported that there was a good correlation between the retention of preservative and the air flow properties of timber. Tesoro *et al* (1966) also showed some relationship between creosote retention and tangential air permeability for various wood species though not Scots pine or Sitka spruce. However given the relationship seen in figure 5.6 it would appear likely that increasing the air permeability of wood samples with *Trichoderma* would increase liquid uptake and hence preservative uptake. The relationship observed in figure 5.6 however was calculated for decalin which will have a lower viscosity than creosote. However it is likely increasing permeability will increase the uptake of the preservative despite differences in viscosity.

Chapter 6 Effect of Wood Extracts on the Growth and Enzymic Activity of *Trichoderma* spp.

6.1. Introduction

Plants were one of the first of the higher levels of organisms to evolve. Their structures have been broken down and recycled by microorganisms for millions of years. Over this time different plant defences against infection by microbes and predation by herbivores have evolved. Different strategies have been developed (Speight and Wainhouse, 1989) and these include physical barriers to infection i.e. impermeable cuticles, physical barriers to predation e.g. thorns and chemical defences to invasion e.g. pinosylvin (Clausen *et al*, 1986).

Generally plants are injured or weakened in some way before microbial invasion of the plant can be initiated. Obviously there are some exceptions to this, but in general, healthy plants will not be easily invaded by micro-organisms. Woody plants are capable of producing a range of chemical defences. e.g. Scots pine can produce chemicals in the families of stilbenes and monoterpenes which are inhibitory to invading organisms.

Man has known of these chemical properties of timbers since early times. Phoenicians were observed to have built their ships from the heartwood of durable timbers which meant that the timbers lasted longer in a potentially hostile environment. The durability of different timbers has long been known to differ between species due to the different chemicals upon which the trees base their defence (BS EN 350-2, 1994).

The timbers used in the following series of experiments are from trees that have different defence mechanisms as well as different inherent durabilities (Hue,1992). Sitka spruce relies on the production of resins as a method of preventing infection. When the tree is wounded resins are produced at the site of wounding which are generally inhospitable for both animals (e.g. beetles) and microbes (Speight and Wainhouse, 1989). One notable

exception to this is *Dendrocinus micans* which is the only beetle at present that can invade Sitka spruce in this country (Bevan and King, 1983). The *Pinus* genus is well documented for the durability of its timber (BS EN350-2, 1994) and also for the production of various compounds at the site of wounding that protects the timber from invasion by microbes and animals (Speight and Wainhouse, 1989; Delorme and Lieutier, 1990; Lieutier *et al,* 1991). However after death or felling the defence mechanisms of some species are lost or become less efficient.

Scots pine sapwood is fairly hospitable for the growth of many fungi and is used as the bench mark timber in decomposition tests (EN113, 1980). The heartwood of this timber is relatively more durable and will resist decay for several years because of the compounds that are stored there during the life of the tree. Consequently the timber will last longer in the environment after the death of the tree. Western red cedar (*Thuja plicata*) however is one of the most durable timbers that is readily available. In the United states it commonly used untreated for roof shingles with life expectancy of 25 years (Scheffer 1957).

Spruce sapwood like pine sapwood is not durable and is readily invaded by microbes. The heartwood material is thought to be more durable than the sapwood material not because of chemical defences like those of pine but because of the different composition of the heartwood material. The carbon to nitrogen ratio in this timber is thought to make the timber relatively inhospitable to decay organisms (Scheffer and Cowling, 1966). Spruce heartwood is however regarded as less durable than the pine equivalent (BS EN350-2, 1994).

If *Trichoderma* isolates are to be able to grow within the timber of these two species then they must be capable of growing in the presence of the chemicals found in these regions. To investigate the effects of these wood types on the growth of *Trichoderma* isolates a series of experiments were conducted to help determine if *Trichoderma* could grow effectively in the presence of some of the chemical extracts found in pine and spruce

sapwood and heartwood, and whether these isolates could grow through the heartwood of these selected timber species. Additionally if *Trichoderma* isolates are expected to act as permeability enhancing agents in these wood types then chemical extracts should have a minimal effect on the degradative enzymes of the fungi.

6.2 Experiment 1: GROWTH OF SELECTED *TRICHODERMA* ISOLATES ON AGAR MEDIUM CONTAINING SAWDUST SAMPLES FROM PINE AND SPRUCE SAPWOOD AND HEARTWOOD.

6.2.1 Introduction

The simplest test to establish whether fungi are able to grow in the presence of selected compounds or materials would be to introduce these into growth medium. By combining sawdust with malt extract agar it was expected that the presence of any toxic extracts in the test material would influence the growth of the isolates. Since Scots pine relies on chemical defence as a barrier to invasion only pine material was investigated in the following experiment.

6.2.2 Methods

Samples of air dried Scots pine sapwood and heartwood were processed in a hammer ball mill so that the material could pass through a 0.5mm mesh. Growth plates were then prepared by placing 5 grams of the sawdust in the sterile plate and adding 10ml of sterile 3% malt extract agar to the sawdust. The sawdust and agar were then mixed with a sterile stirring rod to ensure that all of the sawdust was dispersed in the agar. By pouring the agar at a hotter temperature than usual it was hoped to eliminate some contamination from surface organisms on the sawdust. If autoclaving had been used to sterilize the sample material there was the possibility of extracts being dissolved out of the wood and reducing the effect of the timber on the growth of the isolates. Prepared plates were inoculated with

agar cores containing one of 5 selected *Trichoderma* isolates (see chapters 3 and 4) and were incubated at either 25 or 22°C depending on the individual isolate. The visible growth of the *Trichoderma* isolates across the plates was measured daily until complete coverage of the plate was achieved.

6.2.3 Results.

The growth rate of the five selected *Trichoderma* isolates on malt extract agar containing untreated Scots pine sapwood and heartwood can be seen in table 6.1.

	Mean Growth rate (mm/day)	
Trichoderma isolate	Scots pine Heartwood	Scots pine Sapwood
T. aureoviride SIWT1	2.30	3.29
T. polysporum	1.67	7.33
T. pseudokoningii SIWT 51	1.33	5.66
T. pseudokoningii SIWT 64	3.66	11.67
T. viride SIWT70	1.33	6.00

Table 6.1 Growth rate of five selected *Trichoderma* isolates on 3% malt extract agar with added Scots pine sapwood and heartwood sawdust.

The results in table 6.1 show that there is a significant reduction in the growth rate of the *Trichoderma* isolates grown on the heartwood medium compared with growth on agar containing sapwood. When these results are compared to those in table 2.3 it is possible to see that in most cases the sapwood material has a slight inhibitory effect on the growth of the isolates.

6.2.4. Discussion

In growing timber the heartwood region can contain complex chemicals that are laid down during growth as either waste compounds or precursors for the defence of the tree (Forrest, 1982). In this experiment it is possible to see that the presence of the heartwood material in the growth medium will reduce the growth rate of the *Trichoderma* isolates grown on such medium. This implies that some of the compounds that are responsible for the inhibition of fungal growth are water soluble and are transferred into the growth medium affecting growth of the organisms. None of the tested organisms showed complete inhibition of growth but the rate of growth on the heartwood material was lower than on the equivalent sapwood material for all isolates. It is notable that not all *Trichoderma* isolates show the same levels of inhibition. Since the wood used in this experiment was air dried it is likely that any inhibition observed must come from compounds that are extracted from the timber by the addition of the hot agar, further investigation of the extractive material that is water soluble and its effect on the growth of *Trichoderma* may give a clear indication of the organisms ability to grow throughout the heartwood and sapwood of the timber.

6.3 Experiment 2: AGAR TESTING OF WATER SOLUBLE WOOD EXTRACTS

6.3.1 Methods

Extract Preparation:

Core samples (70mm long, 23mm diameter) were removed from freshly felled and air dried Scots pine and Sitka spruce logs. Sapwood and heartwood samples of each species were milled separately to pass a 0.5 mm mesh. Each wood sample (4 grams) was placed in pre-dried cellulose crucibles and leached in soxhlet apparatus with distilled water (200mls) for 21 hours. The resulting extract was then sterilised by filtration through a sterile membrane (pore size 0.45 mm).

Agar plate test system:

Equal volumes (10 ml) of sterilized malt extract agar (Oxoid CM59) and sterile wood extract were combined to form a solid growth medium in vented petri dishes. Control plates were prepared as above with sterile distilled water being used in place of the extract. Sets of the extract and control plates (six replicates for each treatment) were inoculated with one of six selected *Trichoderma* isolates (*Trichoderma aureoviride* (SIWT.1), *Trichoderma polysporum* (IMI 206039), *Trichoderma pseudokoningii* (S.I.W.T. isolate 51), *Trichoderma pseudokoningii* (S.I.W.T. isolate 64), *Trichoderma viride* (S.I.W.T. isolate 70), *Trichoderma viride* (S.I.W.T. isolate 100), and incubated at either 22 or 25°C. The length of hyphal extension by the isolates across the agar was measured daily. The degree of inhibition was established by comparing the growth of the selected *Trichoderma* isolates on plates containing wood extracts to that on control plates. Growth inhibition was then expressed as the difference in mean hyphal extension in presence and absence of wood extracts as a percentage of the latter after 3 days incubation.

Biomass Test system:

Equal volumes (10 ml) of a minimal medium broth based on that used by Freitag (1989) and sterile wood extract were combined to provide a liquid growth medium in vented petri dishes. Control plates were prepared as above with sterile distilled water being used in place of the extract. Sets of the extract plates (six replicates) were inoculated with the same *Trichoderma* isolates used in the agar plate system and incubated at either 22 or 25°C for 7 or 15 days. Half of the plates were harvested after 7 days and the remainder after 15 days. The mycelia were separated from the growth medium by filtration onto pre-dried and pre-weighed filter papers and the dish washed with distilled water to ensure that all of the mycelia was transferred onto the filter paper and that the medium was washed from the mycelial mat. The papers were dried overnight at 103°C and then re-weighed and the dry weight of fungal mycelium recorded. The results of this system were expressed in terms of the inhibition of the isolates compared with controls.

$$\frac{\text{(Dry weight of control mycelia - Dry weight of mycelia in presence of extract)}}{\text{Dry weight of control mycelia}} \times 100$$

= % inhibition of *Trichoderma* isolates grown in the presence of different wood extracts.

6.3.2. Results

6.3.2.1 Hyphal Extension of Selected Isolates on Small Agar Plates

The % inhibition of *Trichoderma* isolates grown on water soluble Sitka spruce extracts combined with malt extract agar is shown in Figure 6.1.

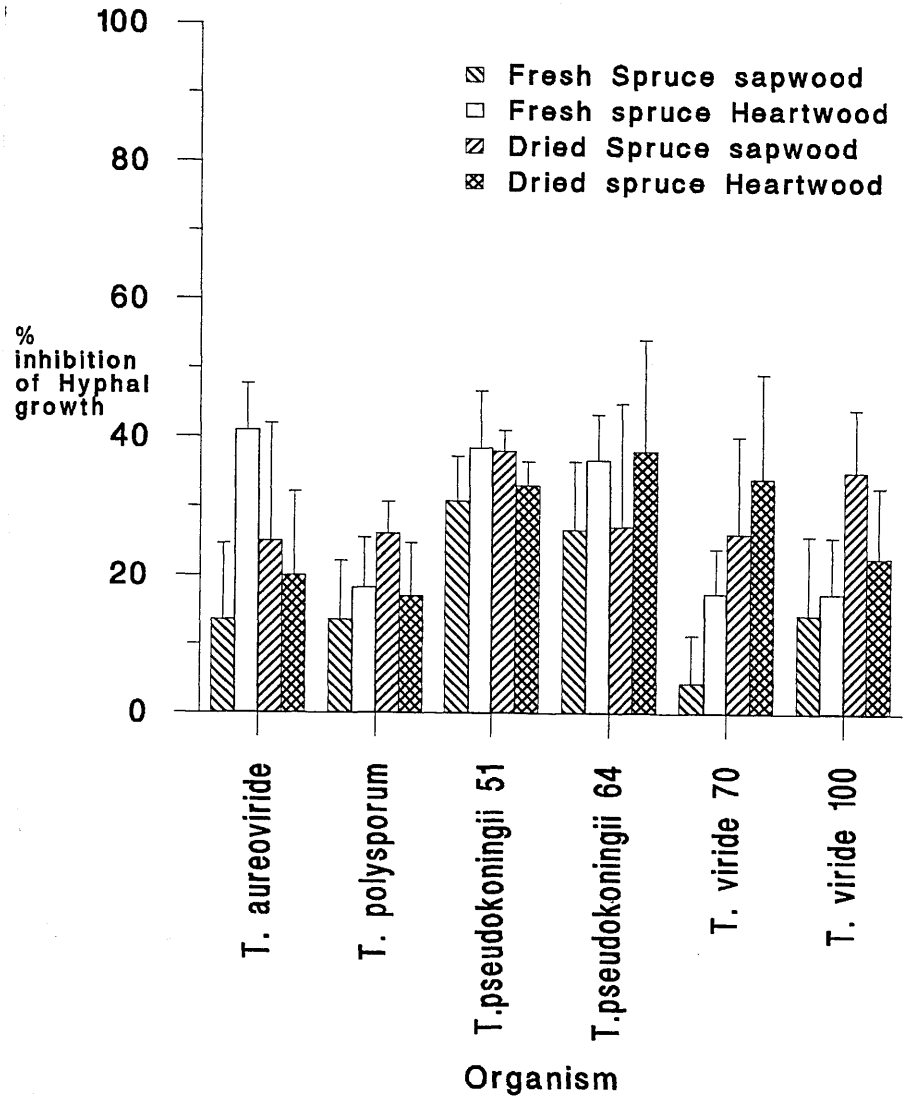


Figure 6.1 The % inhibition of hyphal extension of selected *Trichoderma* isolates grown on Sitka spruce sapwood and heartwood extracts. N.B. Error bars were calculated as the standard error of the data set.

The growth of all *Trichoderma* isolates was inhibited by wood extracts. Although there is considerable variation between the different isolates there are no consistent differences between fresh and dried or sapwood and heartwood extracts.

The effect of wood extracts on *Trichoderma* isolates when grown on malt extract agar including Scots pine extract is shown in Figure 6.2.

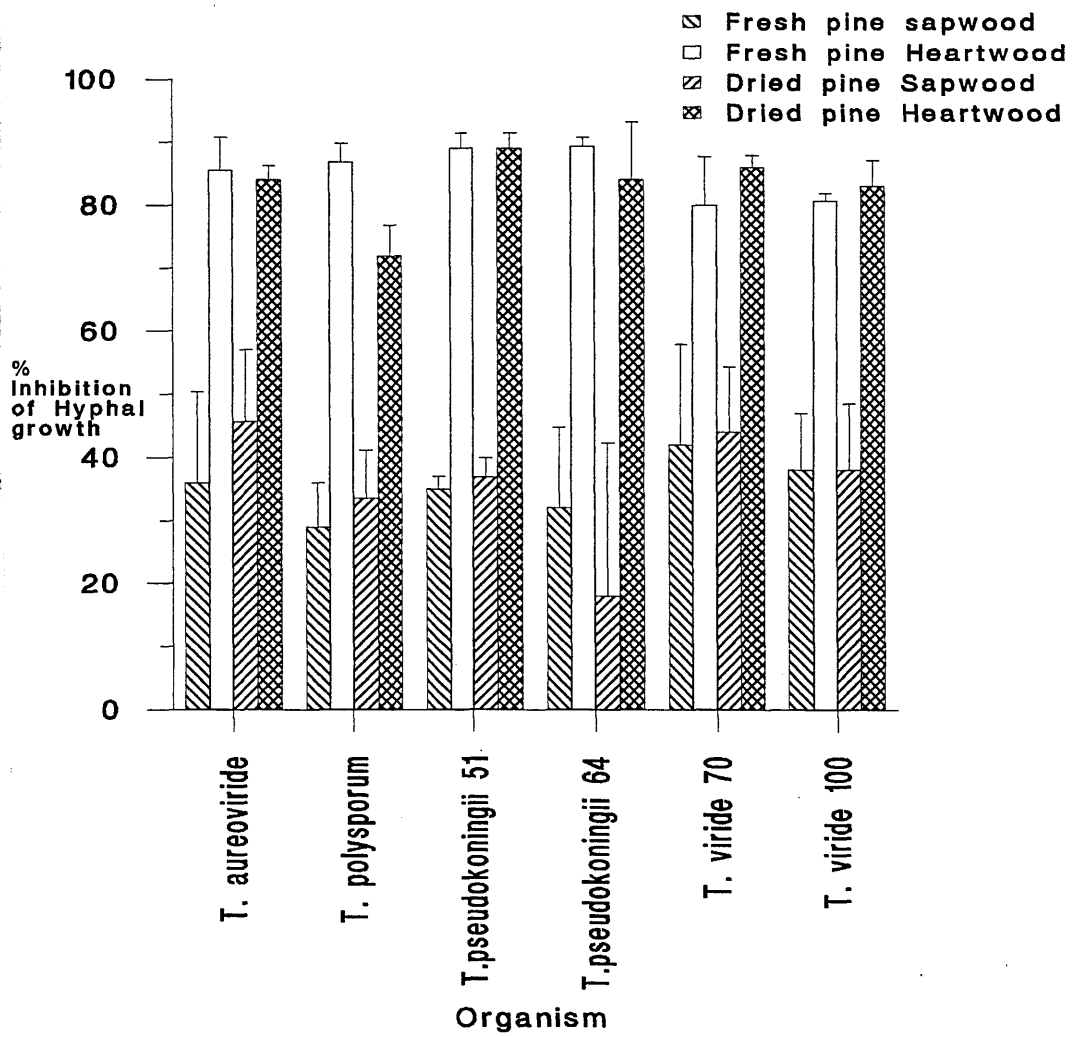


Figure 6.2 The % inhibition of hyphal extension of selected *Trichoderma* isolates grown on Scots pine sapwood and heartwood extracts. N.B. Error bars were calculated as the standard error of the data set.

Although levels of inhibition in Scots pine sapwood are broadly similar to those found in Sitka spruce (Figure 6.1) inhibition by heartwood extracts of Scots pine are significantly higher. Again as with Sitka spruce there are no evident differences between the fresh and dried extracts. Unlike the Sitka spruce extracts however all *Trichoderma* isolates appear to be affected to a similar degree by the Scots pine extracts although in no instance were the *Trichoderma* isolates totally inhibited.

6.3.2.2 Biomass Results

The results from the biomass experiment for Scots pine and Sitka spruce are shown in Figures 6.3 and 6.4 respectively. The percent inhibition of the selected isolates after growth for either 7 or 15 days on Scots pine extract is shown in Figure 6.3.

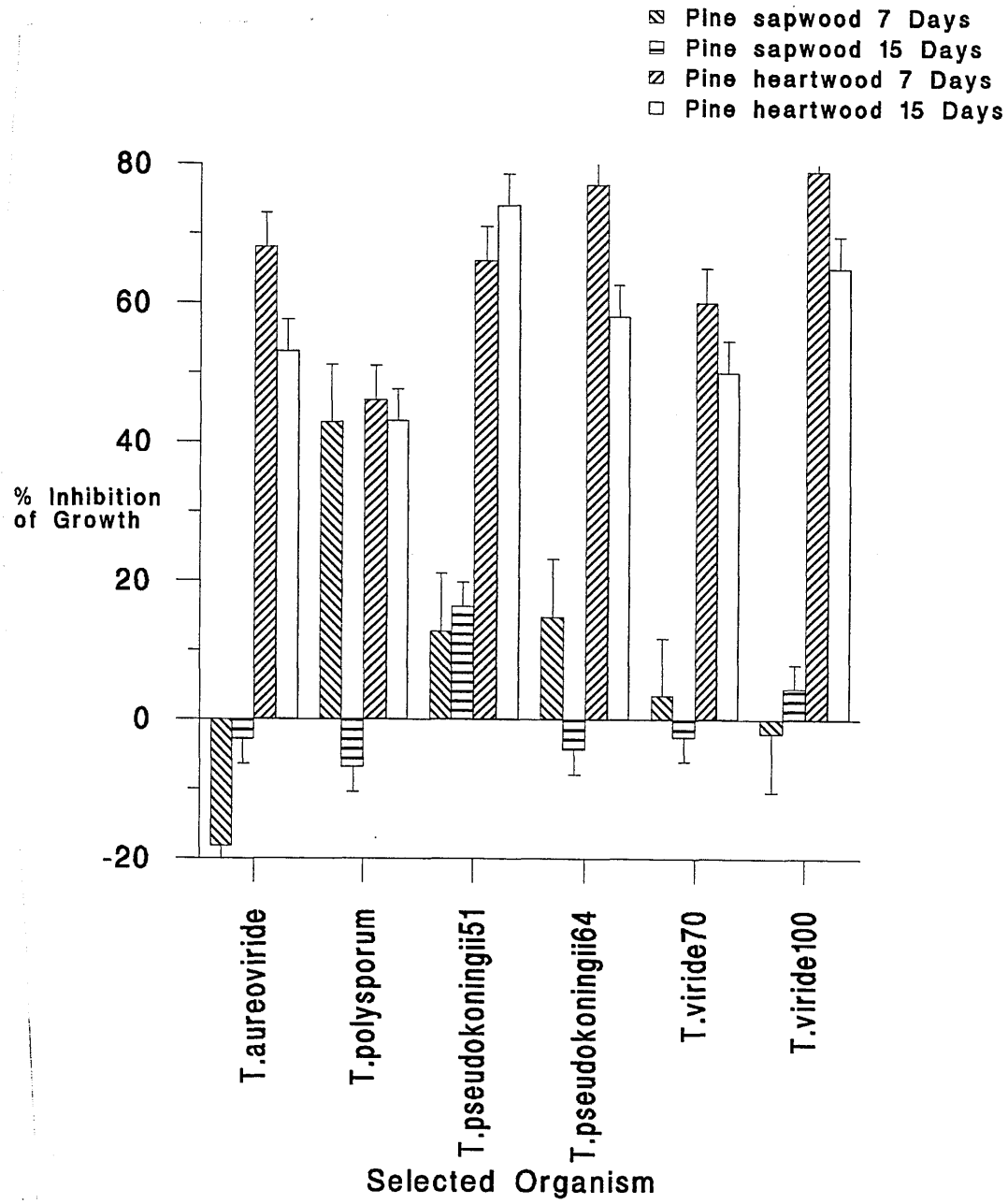


Figure 6.3 The % inhibition of biomass of selected *Trichoderma* isolates grown on Scots pine sapwood and heartwood extracts. N.B. Error bars were calculated as the standard error of the data set.

As with the agar plate system, heartwood extracts inhibited the growth of the fungi to a much greater extent than the sapwood extracts, albeit levels of inhibition are lower than those recorded using the agar plate test system. For heartwood extracts however the level of inhibition is reduced in most *Trichoderma* isolates after 15 days compared with results at 7 days. This would appear to indicate that the extracts have fungistatic activity (a stalling effect) on the growth of the *Trichoderma* isolates.

The % inhibition of the selected isolates after growth for either 7 or 15 days on Sitka spruce extract can be seen in Figure 6.4.

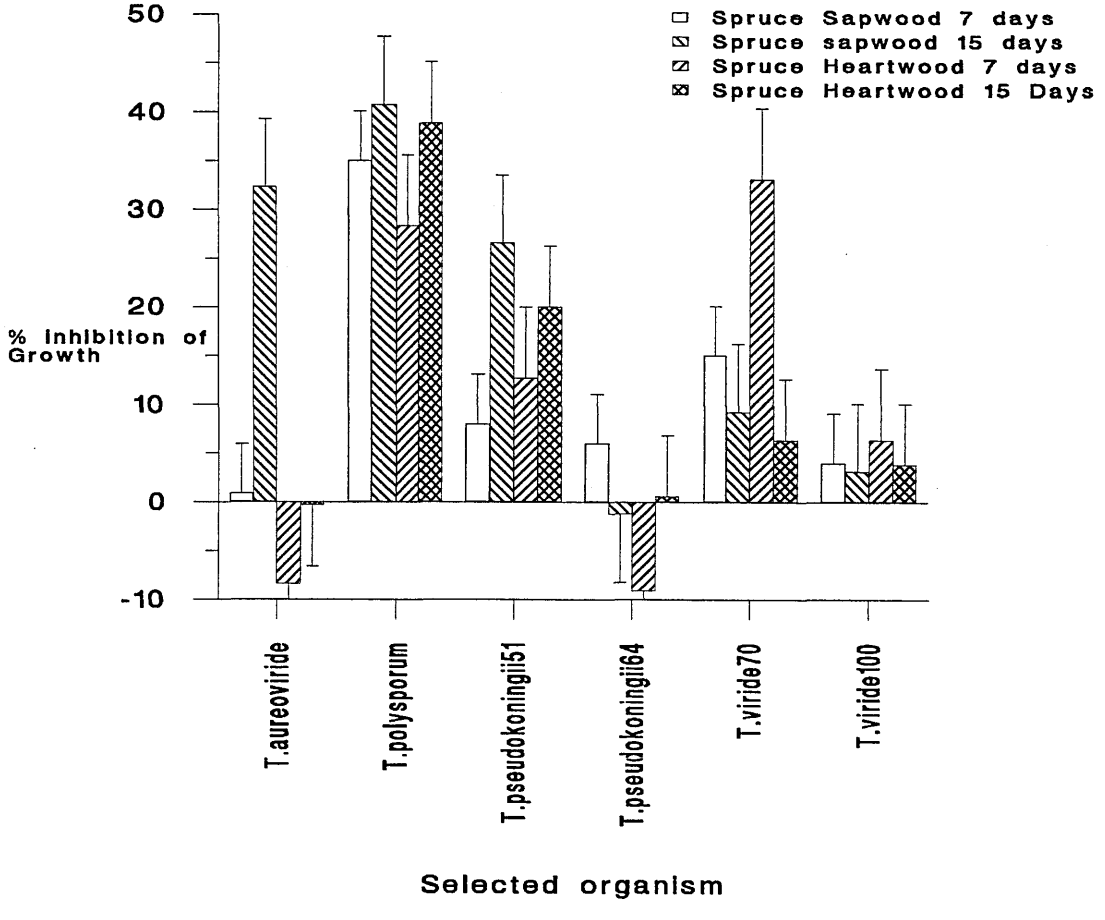


Figure 6.4 The % inhibition of biomass of selected *Trichoderma* isolates grown on Sitka spruce sapwood and heartwood extracts. N.B. Error bars were calculated as the standard error of the data set.

As in the agar plate test system the inhibition of the *Trichoderma* isolate by both the fresh and dried extracts of Sitka spruce were significantly lower than those recorded for Scots pine and again showed large inter-strain variation in sensitivity. However on the liquid medium there was a much greater variation in the levels of inhibition caused by the spruce heartwood and sapwood extracts. Two organisms showed slightly stimulated biomass production in the presence of these extracts.

All of the test plates showed that biomass of selected *Trichoderma* isolates was still increasing after 7 days incubation with greater biomass obtained on harvesting the isolate plates after 15 days.

6.3.3 Discussion

Extractives are generally located in the heartwood region of the living trees with some sap soluble extractives being transported in the sapwood. On felling and drying, the majority of these extractives remain fixed in the heartwood region of the timber although the sap-soluble extractives are deposited in the sapwood region of the timber (Browning, 1975). The extractive content of wood may depend on: tree species; genetic differences between individual trees; different tree sizes; and site climate and geography (Scheffer and Cowling, 1966; Hafizoglu,1983). These factors therefore give rise to variation in extractive content between different species and stems within forest stands. Within the tree, extractive concentrations are seen to vary with height and across the diameter of the tree with the largest concentrations being found at the heartwood/ sapwood boundary

(Rudman and Da Costa, 1958). This "in tree" variation in distribution may be due to natural oxidation, biological detoxification of heartwood extractives or continued polymerisation of extractive material (Zabel and Morrell, 1992).

The heartwood region contains a mixture of organic compounds and the effects of these different compounds on the growth of microorganisms has been shown to vary from tree species to species. The heartwood of *Pinus* species is moderately resistant to fungal decay (Hue, 1992) due mainly to pinosylvin and pinosylvin monomethyl ether content (Erdtman, 1949). Pinosylvin is found in the pine heartwood and is associated with wound sites in trees. This and other extractives (e.g. monoterpenes) appear at wound sites and some of these compounds have been shown to prevent the colonisation by some decay fungi (Lewisjohn *et al*,1992). Other volatile wood compounds have also been shown to stimulate the growth of different wood fungi (Glasare, 1970; Mowe *et al*, 1983). Although few stimulative effects on growth of *Trichoderma* were recorded during this study, these may be due to the increased sugar content of the medium due to inclusion of wood sugars freed by the soxhlet extraction.

When extracts were incorporated into malt extract agar and inoculated with different *Trichoderma* isolates during this study all isolates showed inhibition. As expected this inhibition was particularly high with pine heartwood material because of the deposition of larger amounts of extractives in this region. Although the extractives removed from 4 grams of wood material were diluted in 200 ml of distilled water in the test system, pine heartwood extractives still inhibited the growth of *Trichoderma* isolates by up to 90% in the agar test system. The results of the biomass study however indicates that at least part of this inhibition may be due to stalling of the growth of *Trichoderma* isolates since the % inhibition decreased with increased exposure of isolates to the extracts (Figures 6.4 and 6.5). This reduction in inhibition may also however be due to detoxification of the extractive components within the medium (Dunlop-Jones *et al,* 1991).

The experiments described here measure only the effects of those extractives that are water soluble and not those soluble in other solvents. Water soluble extracts are likely to be more important, however, in determining whether an organism will grow in heartwood material, since the water solubility of such extracts may increase their likelihood of being transported across the cell membranes of organisms. Carey *et al* (1984) showed that soaking/ leaching timbers could reduce the decay resistance of Scots pine and this shows that at least some of the components that give Scots pine its durability are water soluble and it is likely that these components are expressed in the agar and broth test systems used here.

Similar systems have been used to test the effects of preservatives and other compounds (including extractives) on the growth rates of selected microorganisms. Results using this type of test system have shown greater inhibition at selected preservative concentrations when compared against test systems employing wood blocks treated to similar preservative loadings (Clark *et al*, 1990; Archer *et al*, 1993). This may be due to the extractives/ preservatives either being more accessible in agar medium or that lower concentrations of extracts leach out of blocks during the experiment. Alternatively extracts may be interacting with the *Trichoderma* and inhibiting their growth and/ or uptake of nutrients. Archer *et al* (1993) found that agar test systems gave more reproducible results than wood block systems because the incorporation of wood into test systems introduces the inherent variability associated with the material.

It was expected that dried wood might give a lower degree of inhibition, since on drying, wood samples may lose extractives as some of these compounds volatilize at high temperatures (Zabel and Morrell, 1992). Drying the wood may also make different components more or less extractable. However the actual amount of dried wood used in the extraction would also be greater than the fresh material used as the moisture content of the dried wood was lower than that of the fresh material and hence although 4 grams of both the fresh and dried material was used for extraction the mass of actual wood in the

dried material used was higher. If drying does affect extractive content then it would be expected that the inhibition of dried wood extracts would be lower than fresh wood extracts, however this was not found to be the case (Figures 6.1 and 6.2). This may simply reflect the fact that on a dry weight basis more dried material was used.

Spruce wood generally showed lower degrees of inhibition than the pine wood, and there was little difference between the effects of the sapwood and heartwood extractives. Extracts from fresh spruce heartwood were more inhibitory than sapwood extracts. With dried spruce extracts however, the results varied depending on the *Trichoderma* isolate. The durability of Sitka spruce heartwood is reported to be due to its high C:N ratio which can be as high as 1250:1 and this rather than its extractive content, may account for poor colonisation in nature by saprophytic microorganisms (Scheffer and Cowling,1966).

If *Trichoderma* isolates are to be used for the enhancement of the permeability of timbers or biological control then these organisms should be capable of growing in the presence of compounds naturally occurring in wood. All of the tested *Trichoderma* isolates grew in the presence of water soluble extractives although the growth rates varied between isolates and extracts. Pine heartwood gave levels of inhibition up to 90% but did not totally prevent the isolates from growing, and active growth was maintained after extended incubation in extracts. With both the solid and liquid systems showing growth of the *Trichoderma* isolates in the presence of heartwood extracts it may be possible for these isolates to grow through freshly felled heartwood, during which they may improve the permeability of the heartwood material. Results from wood colonisation experiments (chapter 4) indicate that some of the strains used in this experiment are indeed able to colonise fresh Scots pine heartwood. Growth through the heartwood will be dependant on the production of the necessary degradative enzymes by the colonising *Trichoderma* isolates and whether their activity is adversely affected by the heartwood constituents. Studies were therefore required to determine the effects of extractives on such enzyme production and activity (see section 6.6).

If *Trichoderma* spp. will grow in the heartwood and sapwood of timber species then the possibilities for biological control using *Trichoderma* isolates to inhibit the growth of basidiomycete organisms which colonise the wood either during seasoning or when in service will be greatly enhanced and may increase the service life of a number of timber products. Smith *et al* (1981) showed that *Trichoderma harzianum* would grow in the presence of water soluble extracts from red maple (*Acer rubrum*) and prevent colonisation of wounds in standing trees by decay organisms. Carey *et al* (1984) showed that Scots pine heartwood lost its decay resistance with time, and bio-control of wood with *Trichoderma* isolates may therefore be used to compensate for the loss of such protection by water soluble extractives.

The antagonistic properties of different *Trichoderma* isolates and their ability to kill basidiomycetes varies between isolates and has also been shown to be dependent on growth substrate composition (Srinivasan *et al*, 1992). Different isolates may therefore be required to protect different wood species. The results of this study also indicate that selection of any such biocontrol agent will also require careful consideration of that isolate's capability to grow in the presence of the extractives likely to be present in the particular wooden material.

6.4. Experiment 3: EFFECT OF EXTENDED INCUBATION ON THE GROWTH OF *TRICHODERMA* ISOLATES GROWN IN THE PRESENCE OF WOOD EXTRACTS.

6.4.1 Introduction

Work in section 6.3 highlighted that wood leached in hot water will release compounds that will inhibit the growth of selected *Trichoderma* isolates. The degree of inhibition is

dependent on the wood type used, and the *Trichoderma* isolate exposed to the leachate. Most of the earlier work has used short term experiments where the isolates were exposed to the extracts for only a few days. While these experiments indicated that there was inhibition they did not determine whether this was caused by the organisms current growth being disrupted (i.e. shocked) by the medium for a short period of time until able to adapt to the new medium or whether the growth of the isolates was more generally restricted by the medium.

The aim of the following experiment was to investigate the effects of hot water extracts on the growth of selected *Trichoderma* isolates on large malt extract agar plates.

6.4.2 Methods

Wood samples were extracted as described in earlier work (section 6.3.1), and 100ml of filter sterilised extract was combined with 100 ml of 3% sterile malt extract agar. The medium was then poured into large 450mm square culture plates. Duplicate plates were produced for pine and spruce sapwood and heartwood and control plates. After the agar had set four lanes were cut in the agar to give isolated strips for the measurement of hyphal extension. Duplicate plates were then inoculated with one of two selected isolates (*Trichoderma aureoviride* SIWT1 and *Trichoderma viride* SIWT 70) by placing inoculated agar plugs at one end of the lane. These isolates were selected on the basis that they had shown the best permeability enhancement of small wood blocks as described in chapter 4. The inoculated plates were then incubated at 22^{o}C and the growth of the isolates measured daily and the % inhibition of growth calculated as seen in section 6.3.1.

6.4.3 Results

The results of these experiments can be seen in figures 6.5-6.15.

Figure 6.5 shows the degree of inhibition observed for *T.viride* on wood extract plates;

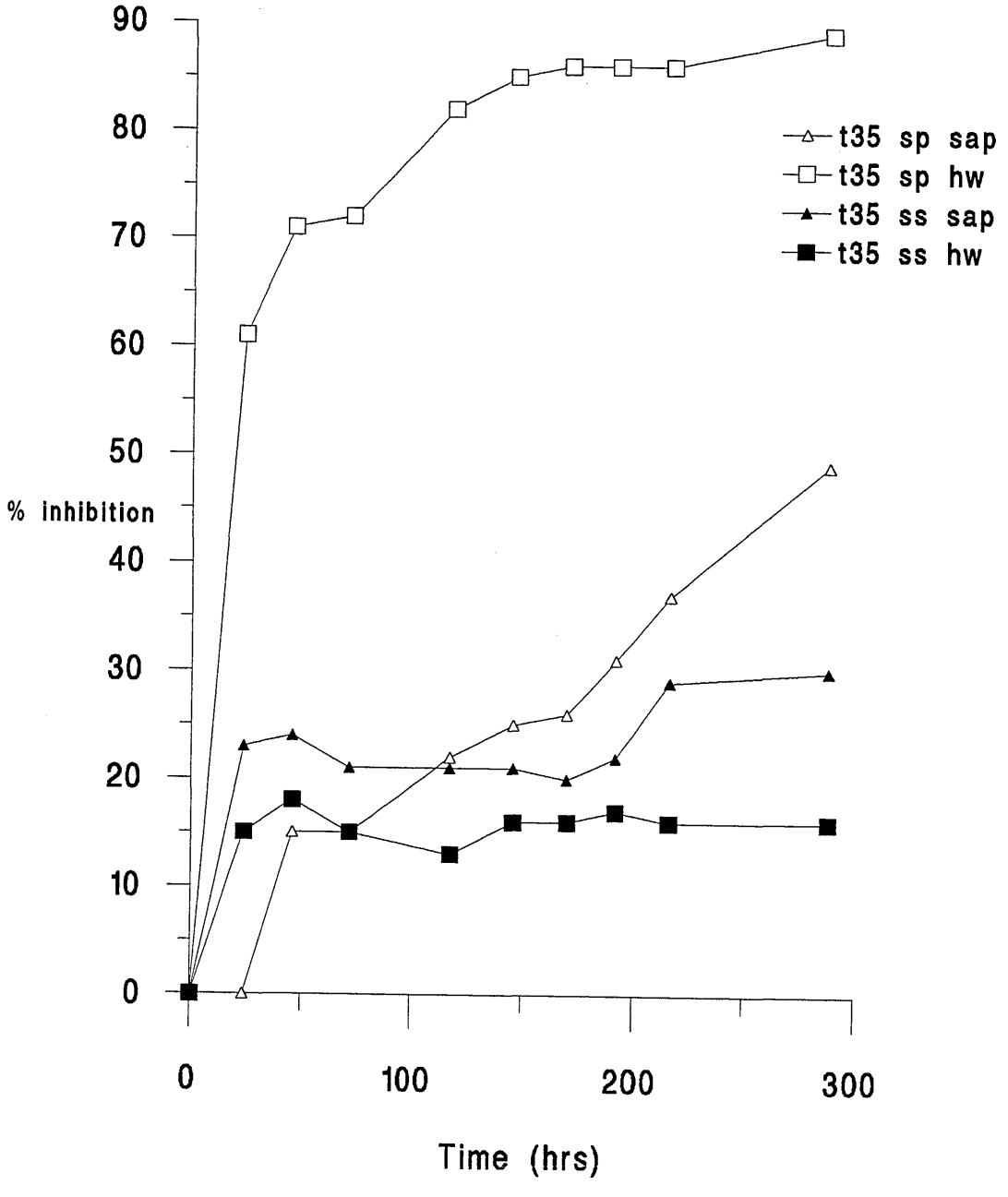


Figure 6.5: Inhibition (%) of *T. viride* SIWT70 (T35) grown on plates containing different wood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

As expected the plates show an inhibition for all of the wood extracts. Pine heartwood shows the highest degree of inhibition throughout the experiment. Patterns of inhibition show that generally the level of inhibition is constant over the duration of the experiment. The exception being with pine sapwood which initially shows no inhibition but this gradually increases over the experiment. Under previous trials this increase in inhibition would not necessarily have been observed as previous agar plate based trials were completed in 96 hours.

The inhibition of *T.aureoviride* SIWT 1 grown on different wood extracts can be seen in figure 6.6.

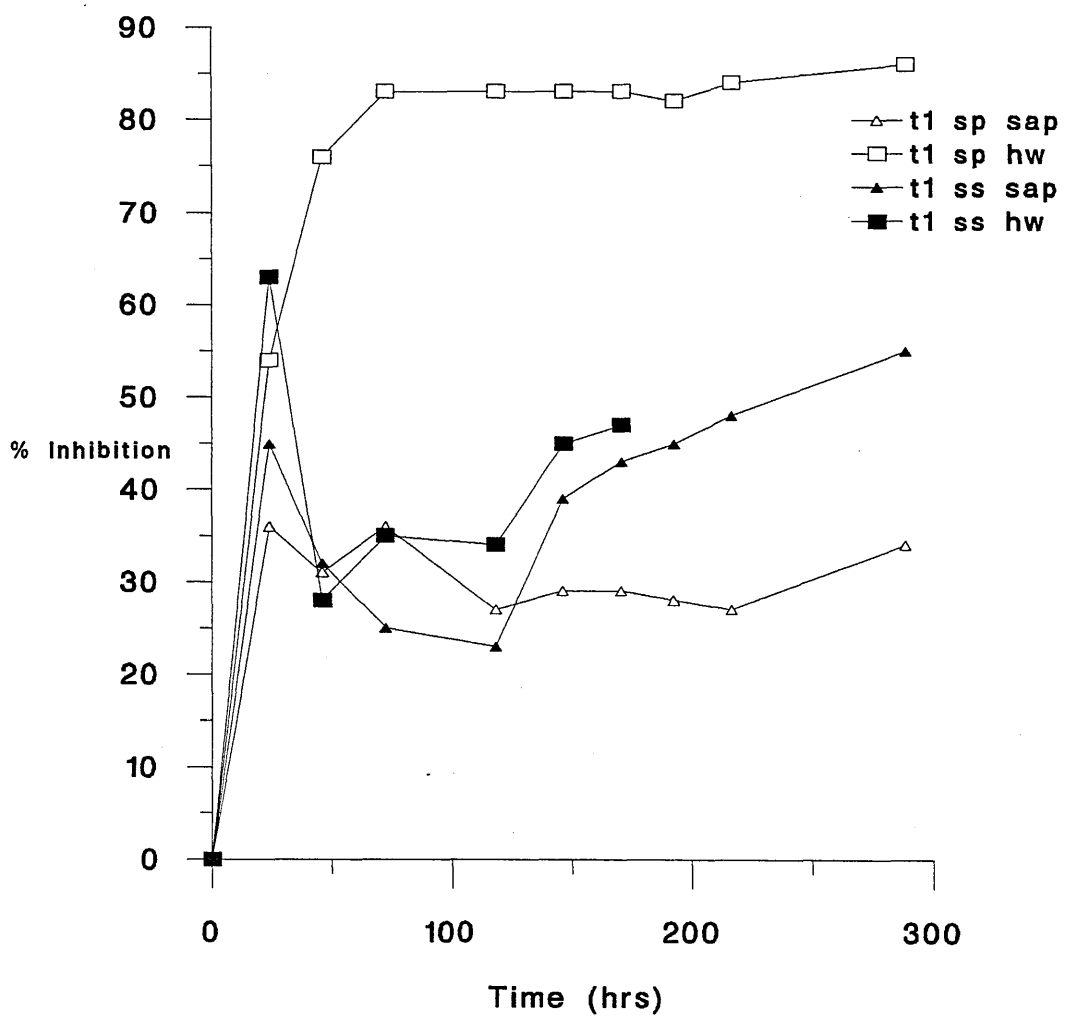


Figure 6.6: Inhibition (%) of *T. aureoviride* SIWT1 (T1) grown on plates containing different wood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

When grown on heartwood extracts *T. aureoviride* SIWT 1 shows the highest degree of inhibition which remains almost constant throughout the experiment. The other extracts show an initial increase and then decline over the first few hours this is likely to be due to shock from the organism being placed on the new growth medium. The inhibition is seen to decline over the next sample periods before increasing once more. This would appear to indicate that the organism is not detoxifying the medium as % inhibition should drop with time as the organism grows more efficiently.

The growth rate of the organisms were measured in terms of mm of hyphae produced per hour on the different medium types. The results of these determinations can be seen in figures 6.7 and 6.8.

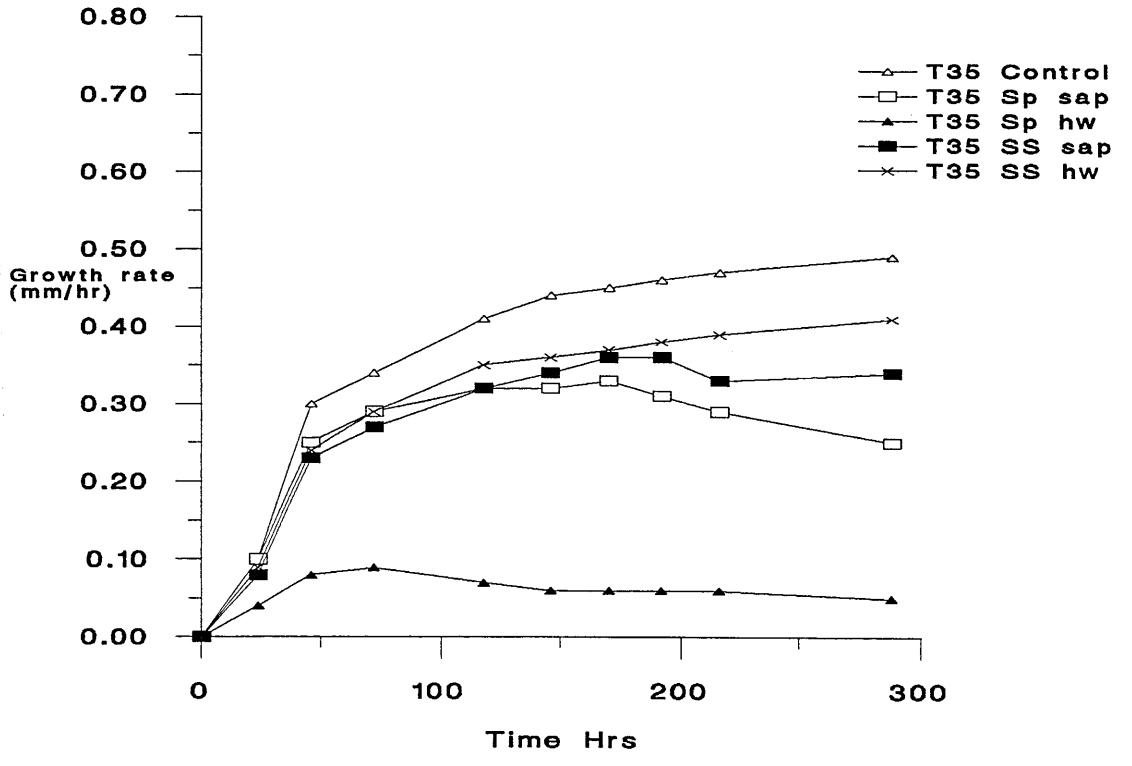


Figure 6.7 Growth rate of *T. viride* SIWT 70 (T35) grown on plates containing different wood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

From figure 6.7 it is possible to see that the initial growth of the organism is exponential, as the experiment progressed the growth rate on the pine sapwood and heartwood medium declined this may be due to the organism being stressed by the medium or showing the following growth characteristics. On the pine heartwood extract it was observed that the growth of the fungal colonies was denser around the original inoculation point and that growth over the agar surface was reduced, this may indicate that the growth medium was stressing the organism as a larger proportion of the hyphae was aerial. Growth on the spruce sapwood and heartwood material was similar to that observed with the control samples albeit the rate was marginally lower.

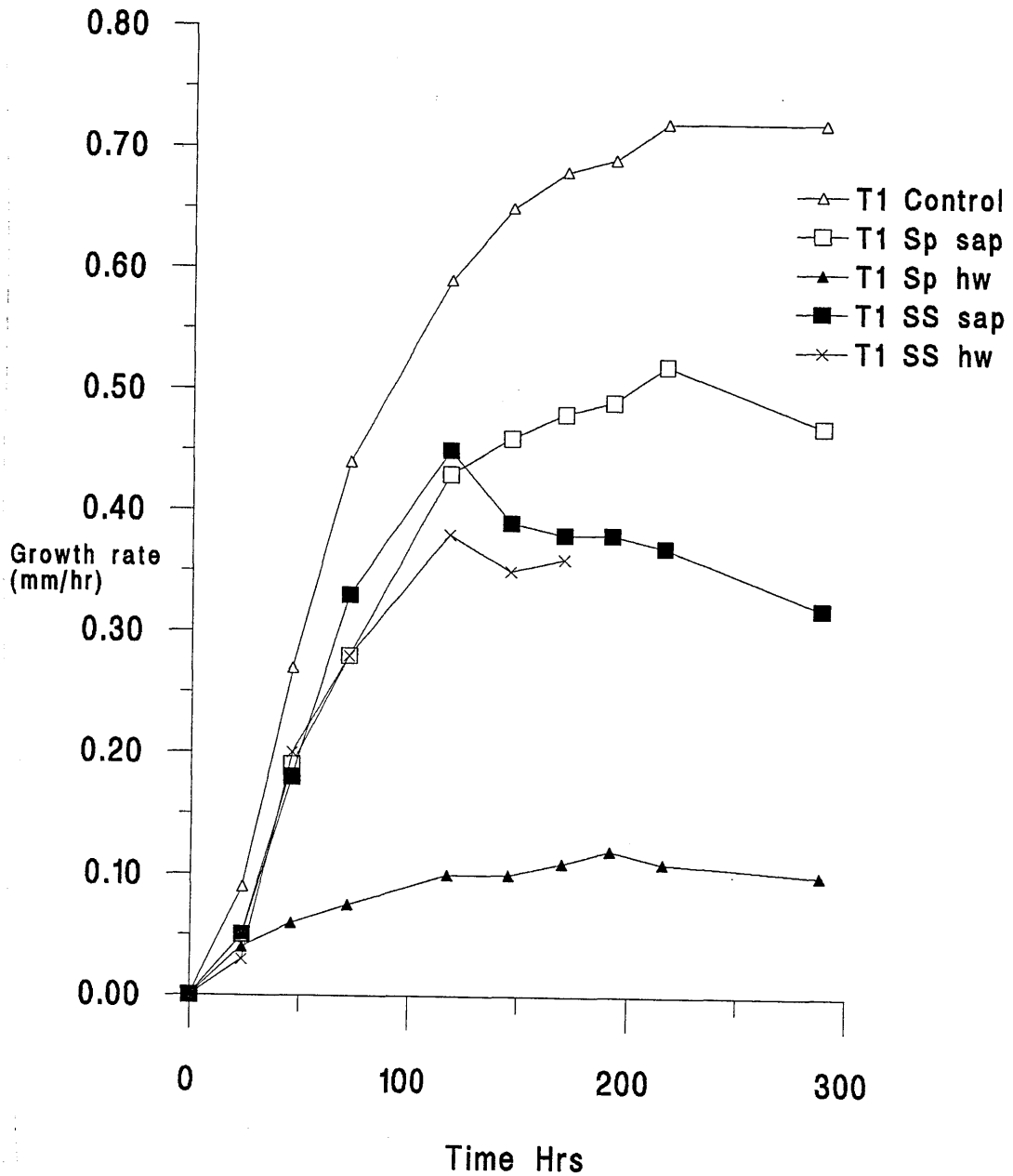


Figure 6.8 Growth rate of *T. aureoviride* SIWT1 (T1) grown on plates containing different wood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The patterns of growth observed in figure 6.8 are similar to those of *Trichoderma viride*. Growth rates again showed the standard growth curve for an organism with initial exponential growth followed by a plateau followed by a decline in growth. There was also a slight lag phase where the spruce sapwood and heartwood and pine sapwood material

showed a lower initial growth rate. The lowest growth rate was again observed on pine heartwood.

The actual hyphal extension for each of the organisms on the different growth medium were plotted against time and the results can be seen in figures 6.9 and 6.10

Figure 6.9 shows the hyphal extension of *Trichoderma viride* when grown on the different growth medium.

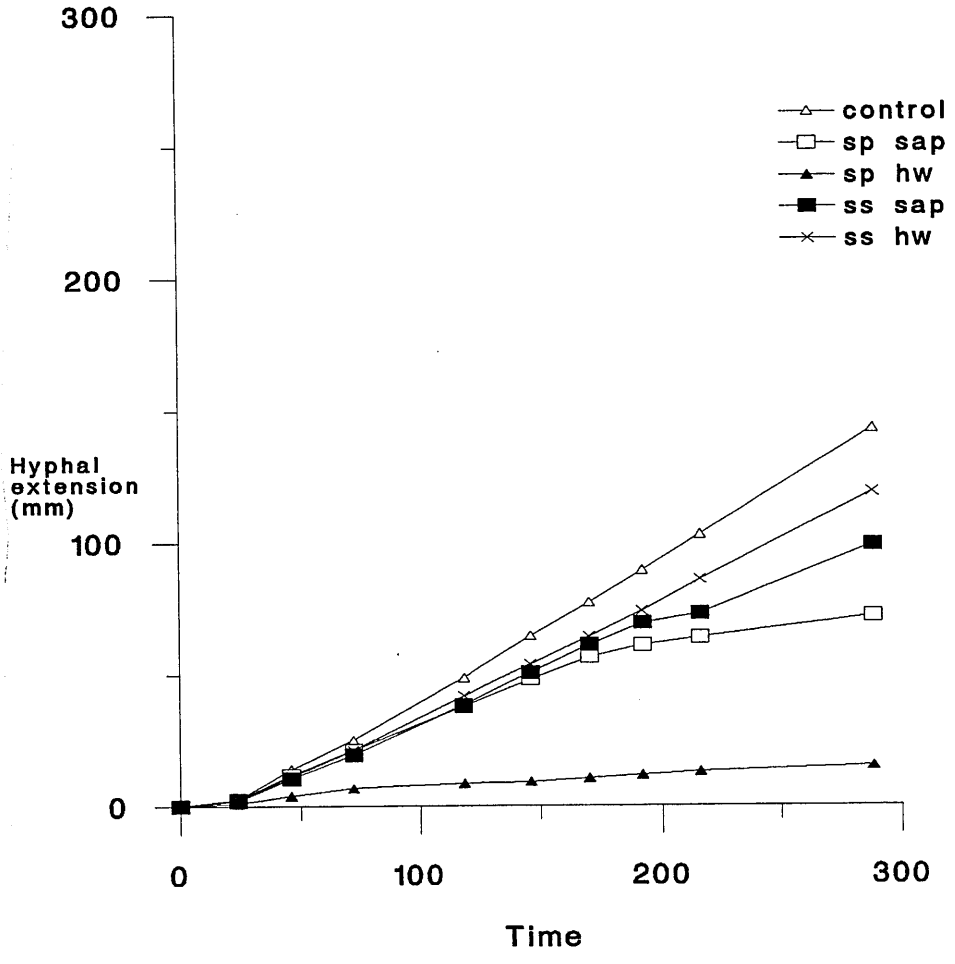


Figure 6.9 Hyphal extension of *T. viride* SIWT 70 grown on plates containing different wood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The growth of *T. viride* on the control and spruce medium shows an almost linear hyphal extension. However when grown on the medium containing the pine extracts a reduced growth rate is seen in later stages of the experiment.

The hyphal extensions of *Trichoderma aureoviride* can be seen in figure 6.10.

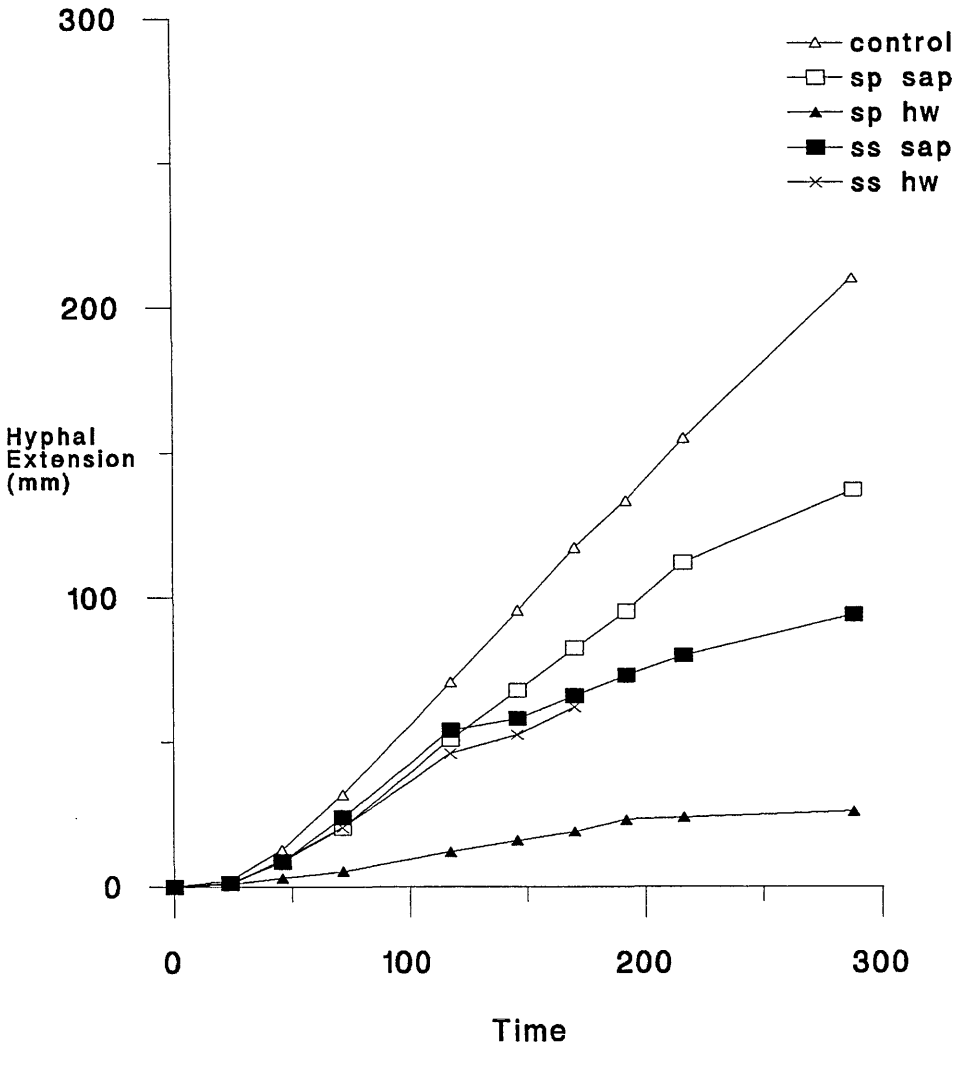


Figure 6.10 Hyphal extension of *Trichoderma aureoviride* SIWT1 when grown on the different growth medium. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The growth of this organism appears to be reduced in the early stages of the experiment i.e. a lag phase occurs. After this lag phase the growth on the control material is almost linear as with *T. viride*.

6.4.4 Experiment 4: EFFECT OF EXTENDED INCUBATION ON THE GROWTH OF *TRICHODERMA AUREOVIRIDE* SIWT1, *T. POLYSPORIUM*, *T. PSEUDOKONINGII* SIWT 51, *T. PSEUDOKONINGII* SIWT 64, *T. VIRIDE* SIWT70 AND *T. VIRIDE* SIWT 100 GROWN IN THE PRESENCE OF WOOD EXTRACTS.

Twenty further plates were prepared as described in section 6.4.2. Each plate contained 3 lanes of agar, a different isolate was inoculated onto each lane. A total of 6 isolates were inoculated onto each set of plates as follows *Trichoderma aureoviride* SIWT1 (T1), *T. polysporium* (T3), *T. pseudokoningii* SIWT 51 (T13), *T. pseudokoningii* SIWT 64 (T15), *T. viride* SIWT70 (T35) and *T. viride* SIWT 100 (T38). The growth on these plates was likewise measured daily.

When groups of three isolates were grown in adjacent lanes in an agar plate on different medium the growth of the isolates were seen to vary. The hyphal extension of these isolates on the different medium is illustrated in figures 6.11-6.15.

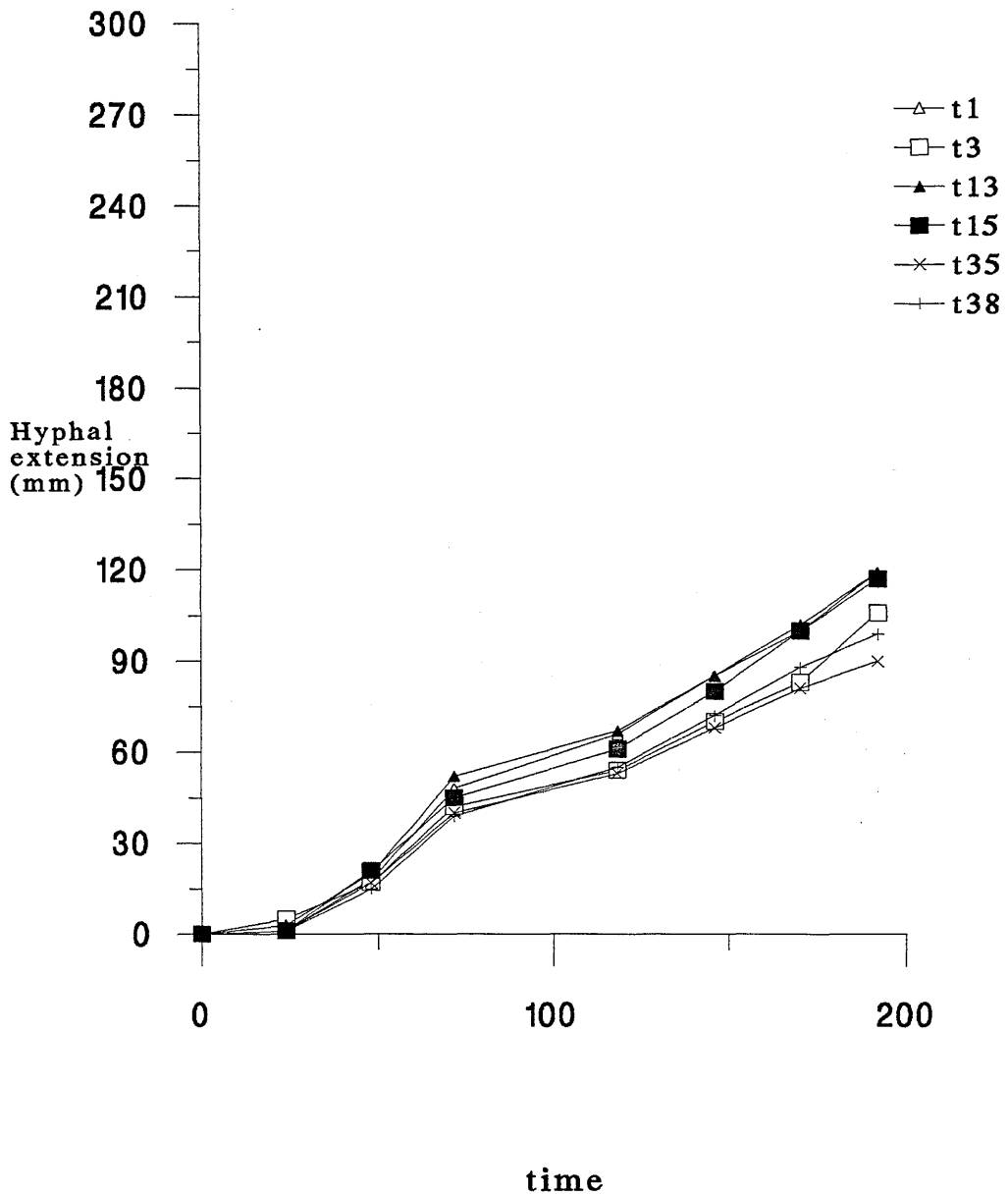


Figure 6.11: Hyphal extension of selected *Trichoderma* isolates grown together on 1.5% malt extract agar. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The results in figure 6.11 show that the initial growth is linear but as the experiment progresses the growth is inhibited compared to the results seen in figure 6.9 and 6.10 This

may be due to the *Trichoderma* isolates interfering with the growth of one another through the production of volatiles.

The effect of pine heartwood extracts on the growth of these same isolates can be seen in figure 6.12.

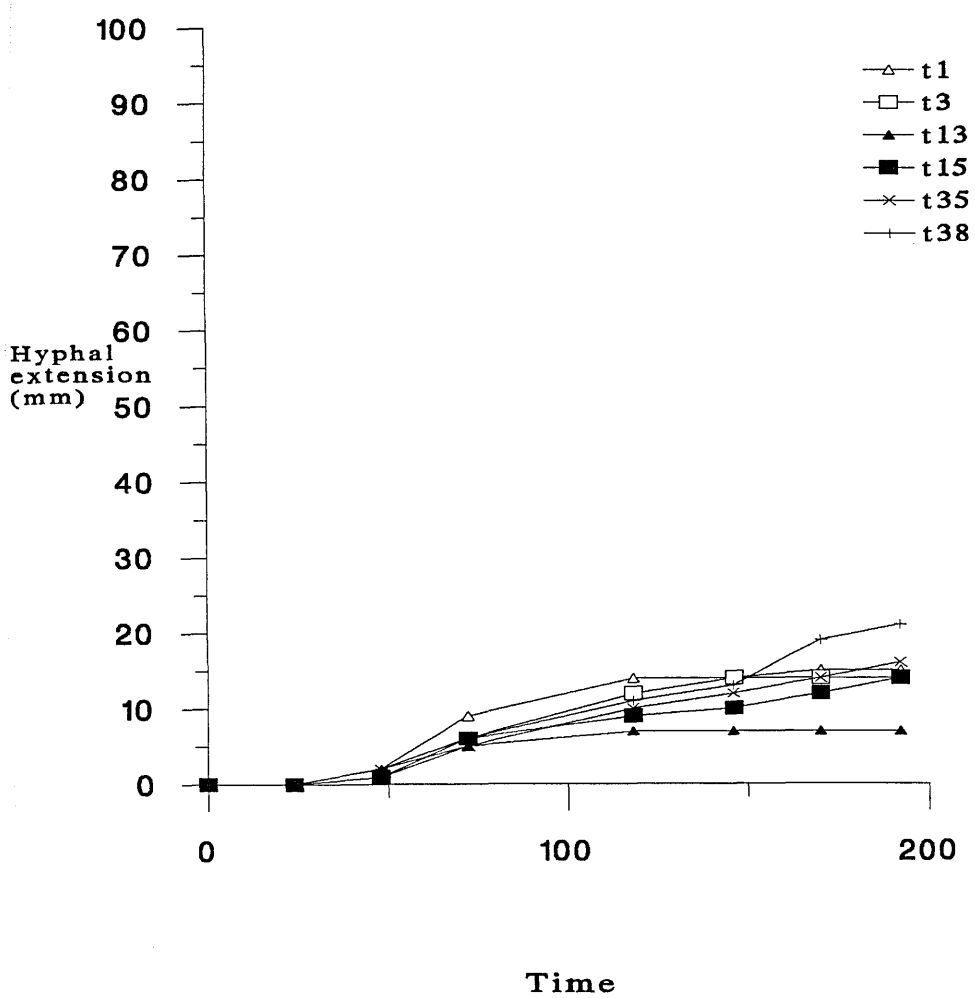


Figure 6.12: Hyphal extension of selected *Trichoderma* isolates grown together on 1.5% malt extract agar containing heartwood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The growth of all isolates was much reduced by the presence of the heartwood extracts even accounting for any inhibition of growth caused by the presence of the other *Trichoderma* isolates.

The pine sapwood results can be seen in figure 6.13

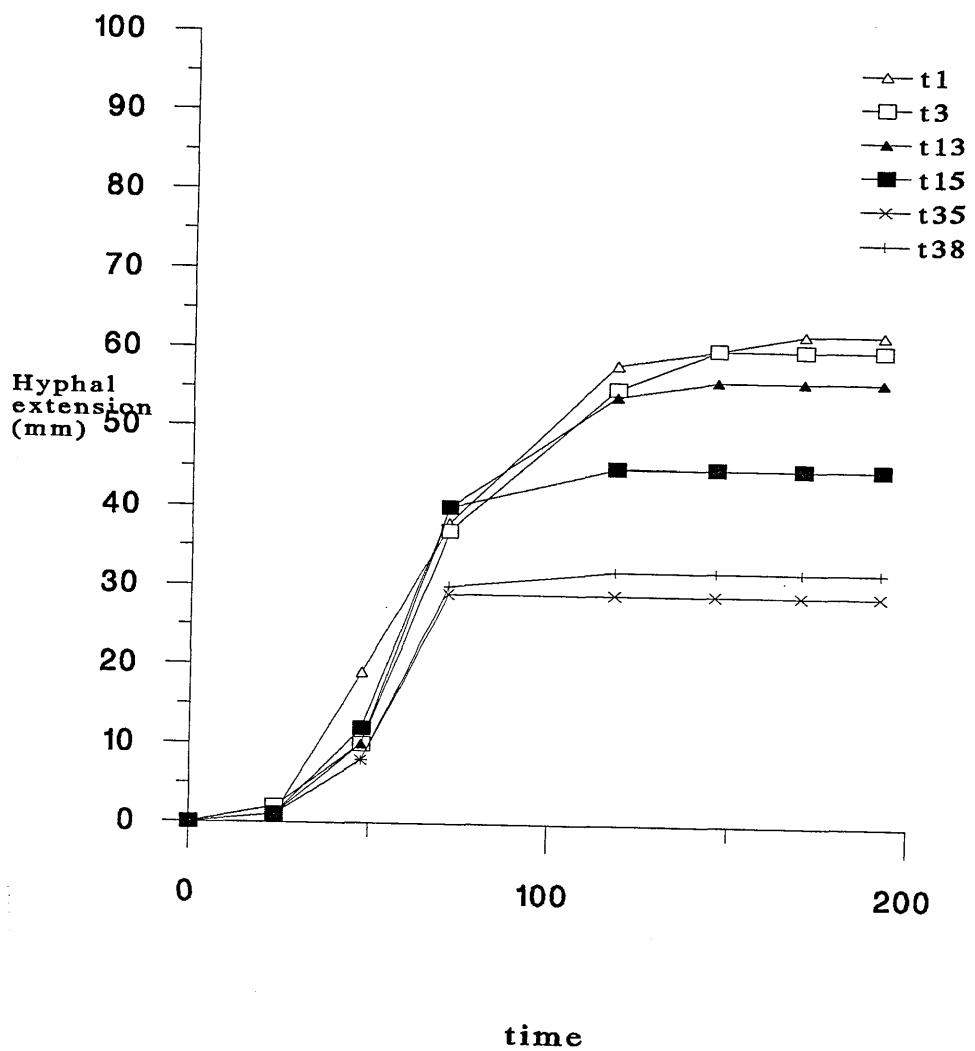


Figure 6.13: Hyphal extension of selected *Trichoderma* isolates grown together on 1.5% malt extract agar containing Scots pine sapwood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. Again when these results are compared to the results in figure 6.9 and 6.10 it can be seen that the organisms have stopped growing This would appear to be caused by the *Trichoderma* isolates inhibiting the growth of each other.

The results for the effects of Spruce sapwood and heartwood on the growth of the *Trichoderma* isolates can be seen in figures 6.14 and 6.15 respectively.

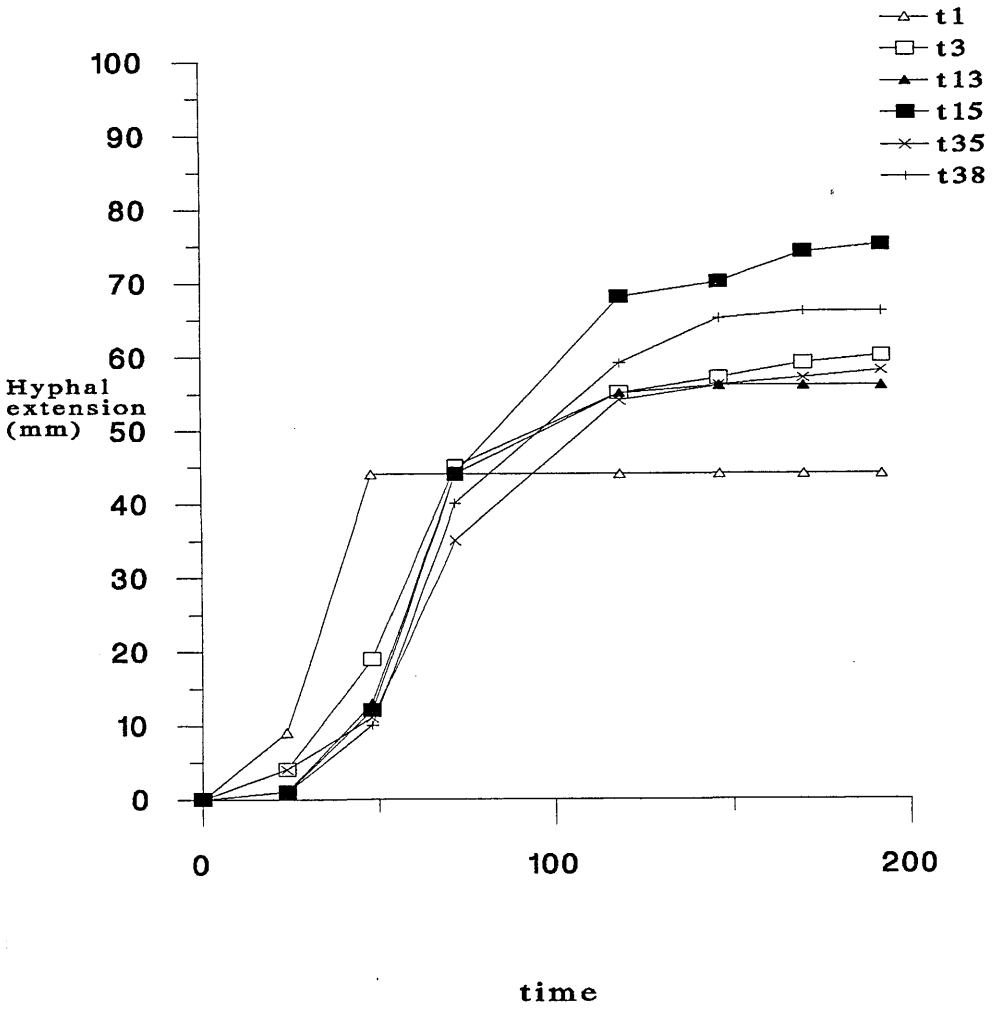


Figure 6.14: Hyphal extension of selected *Trichoderma* isolates grown together on 1.5% malt extract agar and Sitka spruce sapwood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

The initial growth of the isolates is inhibited by the presence of the extracts in the growth medium, but as the experiment progresses the growth of the isolates appears to be further inhibited by the presence of the other isolates on the growth medium. When these results are compared to the earlier experiment it is again apparent that the *Trichoderma* isolates are affecting the growth of the other organisms.

When grown on medium containing spruce heartwood extracts similar results were observed and these can be seen in figure 6.15.

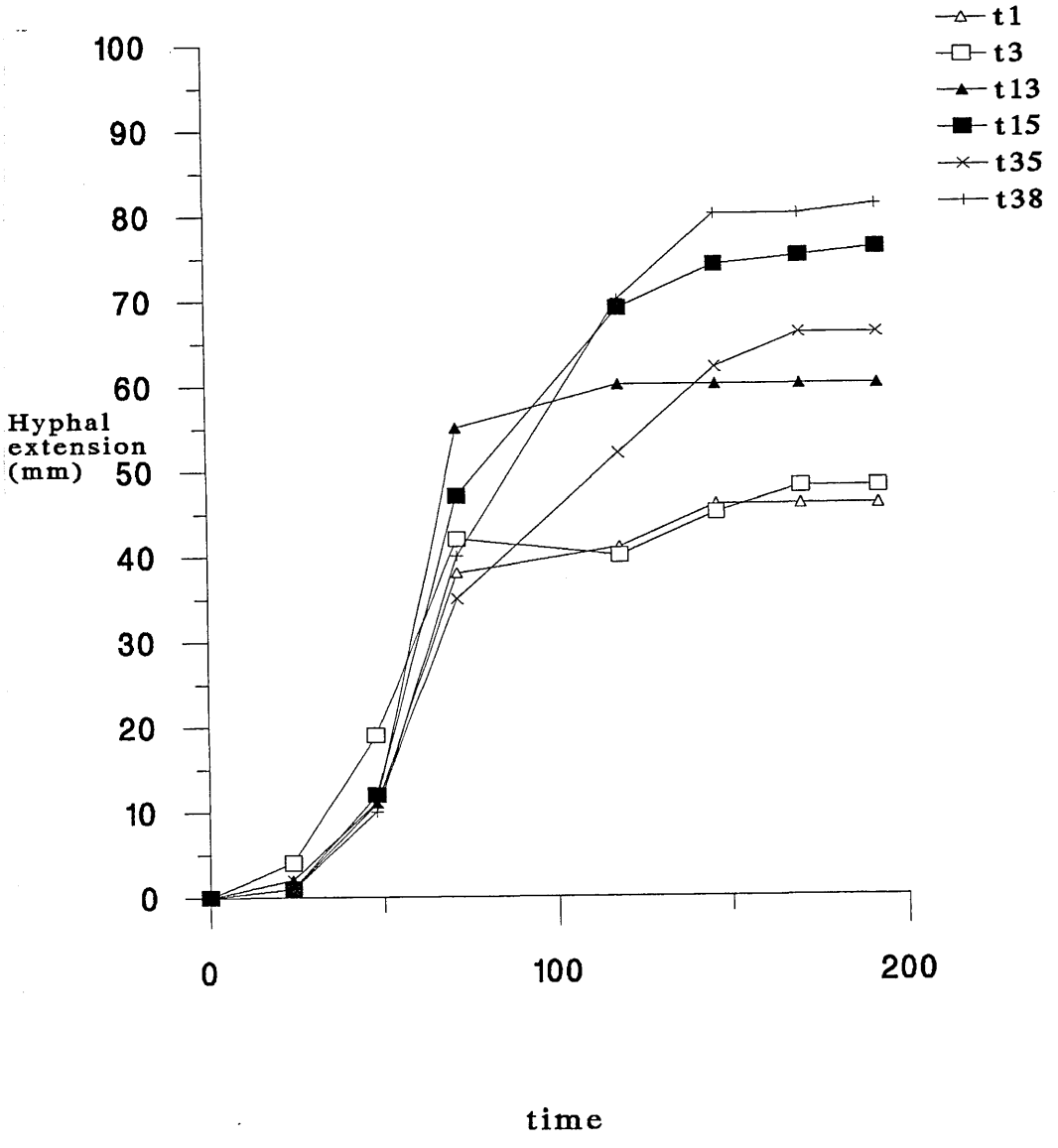


Figure 6.15: Hyphal extension of selected *Trichoderma* isolates grown together on 1.5% malt extract agar containing spruce heartwood extracts. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data.

6.4.5 Discussion

The effects of longer term exposure to the wood extracts appear to be similar to those seen in earlier experiments. The introduction of these different *Trichoderma* isolates onto the same plates however resulted in increased inhibition in the later stages of the experiment for all medium types as can be seen in figures 6.11-6.15. The effect of *Trichoderma* isolates on the growth of other fungi is well documented (Papavizas 1985; Srinivasan *et al*, 1993) although all of the mechanisms involved in the inhibition of the other fungi have not been fully researched (Srinivasan,1993). The production of volatile compounds by *Trichoderma* spp. has been shown to affect the growth of different fungal isolates (Summerbell, 1987; Bruce *et al,* 1996; Wheatley *et al*, 1997). In this experiment it appears that the different *Trichoderma* may be affecting the growth of the other isolates. This may have important implications for bio-control as selected isolates may be affected by *Trichoderma* isolates already present in the environment in which the agent is being used.

With respect to the ability of selected *Trichoderma* isolates to improve the permeability of green timber, it is unlikely that interference of other isolates will have a major effect on the growth of the selected isolates. Since inoculations are made on fresh sites where competition between the selected isolates and other organisms is limited it is unlikely that they will have any effect on the colonisation of the timber by the selected organisms.

The results of the extended growth experiment also show that isolates demonstrate the classic growth pattern (Lag-Exponential- Decline) as described by Gause (1932). The lag phase was apparent on growth medium containing Scots pine sapwood extract where the growth rate is slower as the *Trichoderma* adapt to the new growth medium. This was apparent from Figure 6.8 where the growth rate over the first 24 hours was slower than the following 96 hour period where growth was almost linear. These growth patterns were not

observed in earlier experiments as the time period of these experiment was too short for as detailed an analysis.

It was thought that *Trichoderma* may probably be able to detoxify the growth medium as it grew over the agar or at least adapt to the new growth conditions and grow more effectively on the growth medium. From the results in figures 6.1-6.10 it is possible to see that the organisms do adapt to medium initially but the growth rates remain reduced (compared to the controls) over the entire experiment indicating that no apparent detoxification occurs.

When the growth of the organisms on pine heartwood extracts was analysed it appeared that the growth from the inoculation points was slower but the growth was more dense and colonies more spherical. This implies that the agar was inhibiting the growth of the isolates and affecting the growth pattern of the isolates. With the thicker growth above the agar surface it appears that more effort was being invested in growth on the initial core medium than on the new agar. This apparent negative chemotropism by aerial mycelia was reported by Mowe (1983), he observed that mycelia would not grow towards wood treated with volatile chemical preservatives. From earlier experiments on liquid growth medium when the inhibition was measured in terms of biomass produced, the levels of inhibition on the pine heartwood was much reduced compared to the solid system. In future experiments the medium should perhaps be inoculated with either plugs cut from the same medium type or with spore suspensions placed into wells cut in the agar to ensure that the fungus is totally reliant on the test medium.

6.5 CONCLUSIONS

These experiments have raised several points that may have important implications for the use of *Trichoderma* isolates as permeability enhancement or biocontrol agents. If *Trichoderma* isolates are to be used in field conditions it will be important to ensure that these isolates are among the first organisms to establish on the desired sites. This could

cause problems in an agricultural situation as *Trichoderma* are soil dwelling organisms and the possibility of competition between the inoculated organisms and naturally occurring isolates could cause problems with the establishment of the desired organisms. The growth of selected *Trichoderma* isolates on the different growth medium again showed that the isolates were inhibited by the wood extracts when compared to control plates. No isolate was completely inhibited by the inclusion of the wood extracts in the medium, although different growth patterns were observed over the experiment.

6.6. Experiment 5: EFFECT OF WOOD EXTRACTIVES ON THE PRODUCTION & ACTIVITY OF EXTRA CELLULAR ENZYMES

6.6.1 Introduction

Results reported in sections 6.2, 6.3 and 6.4 have shown that the growth of *Trichoderma* isolates can be inhibited by the addition of either sawdust or hot water wood extracts to the growth medium. The *Trichoderma* isolates selected for experiments to improve the permeability of the wood samples were chosen on the basis of their ability to produce selected extra cellular enzymes. If *Trichoderma* isolates are to improve the permeability of the timber then they must be able to produce these extra-cellular enzymes in the presence of wood extracts and such extracts must not inhibit the action of these enzymes on the target substrates. The aim of this section was to investigate the effect of hot water extracts from Scots pine and Sitka spruce sapwood and heartwood on the production and action of extra cellular pectinase, cellulase and amylase.

6.6.2 Methods.

Wood samples were extracted with hot water in soxhlet apparatus as described for earlier work. (Section 6.3.1). The different extracts (Scots pine and Sitka spruce sapwood and

heartwood) were combined with equal volumes (10 ml) of sterile low nutrient medium (Sections 2.2.2 and 2.3) containing 0.5% pectin, 0.5% cellulose or 0.5% starch. Controls were prepared by replacing the wood extract with sterile distilled water. Selected *Trichoderma* isolates (*Trichoderma aureoviride* SIWT 1 and *Trichoderma viride* SIWT 70) were inoculated into the medium containing either pectin, cellulose or starch and incubated at 22 or 25^{o}C for 4 days (3 replicates were prepared for each treatment). The media were then filtered onto pre-dried and weighed filter papers and the filtrate was retained for analysis. The filter papers were then dried to determine final biomass. The filtrates were then analysed using the same enzyme assay methods as described in (chapter 2).

6.6.3. Results

The following results (figures 6.16-6.22) show the enzyme activity and biomass of two *Trichoderma* isolates grown on medium containing wood extracts and different carbon sources.

6.6.3.1 Pectinase Activity.

The biomass and pectinase activity of *Trichoderma aureoviride* SIWT 1 (T1) when grown on low nutrient medium containing 0.5% pectin and various types of wood extract can be seen in figure 6.16.

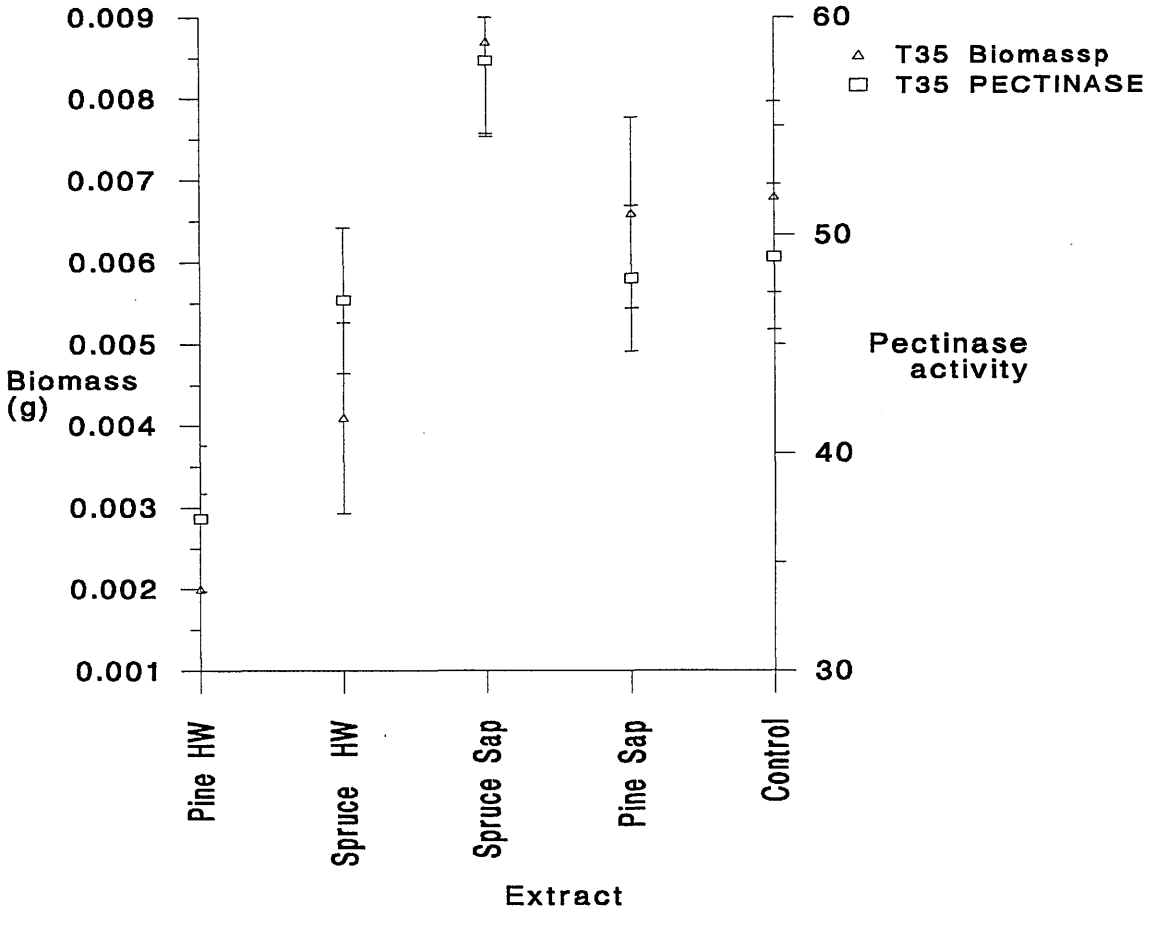


Figure 6.16 Pectinase activity of *Trichoderma aureoviride* SIWT 1 (T1) and biomass from low nutrient medium containing pectin and wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The results in figure 6.16 show that the growth of the *Trichoderma* in the pectin medium containing spruce sapwood extract is stimulated, while in the presence of spruce

heartwood extract growth seems to be inhibited. Pectinase activity of this isolate varies depending upon which extract is present in the growth medium. Media containing larger amount of sugar from the wood extracts (Figure 6.22) show lower enzyme activity i.e. Scots pine sapwood and heartwood extracts but the biomass is similar or greater to the control material. This indicates that the production of the enzyme during incubation may be reduced by the presence of other carbon sources (i.e. Catabolite repression). Spruce heartwood extracts show a low biomass but higher enzyme activity. This may be due to the growth of the organism being inhibited by the extract, however the isolate was capable of producing significant amounts of the enzyme to break down the pectin in solution. Spruce sapwood extracts appear to show a slight stimulatory effect on the growth of the organism in terms of biomass and enzyme activity when compared to the control material.

The biomass and pectinase activity of *Trichoderma viride* SIWT 70 (T35) when grown on low nutrient medium containing 0.5% pectin and various types of wood extract can be seen in figure 6.17.

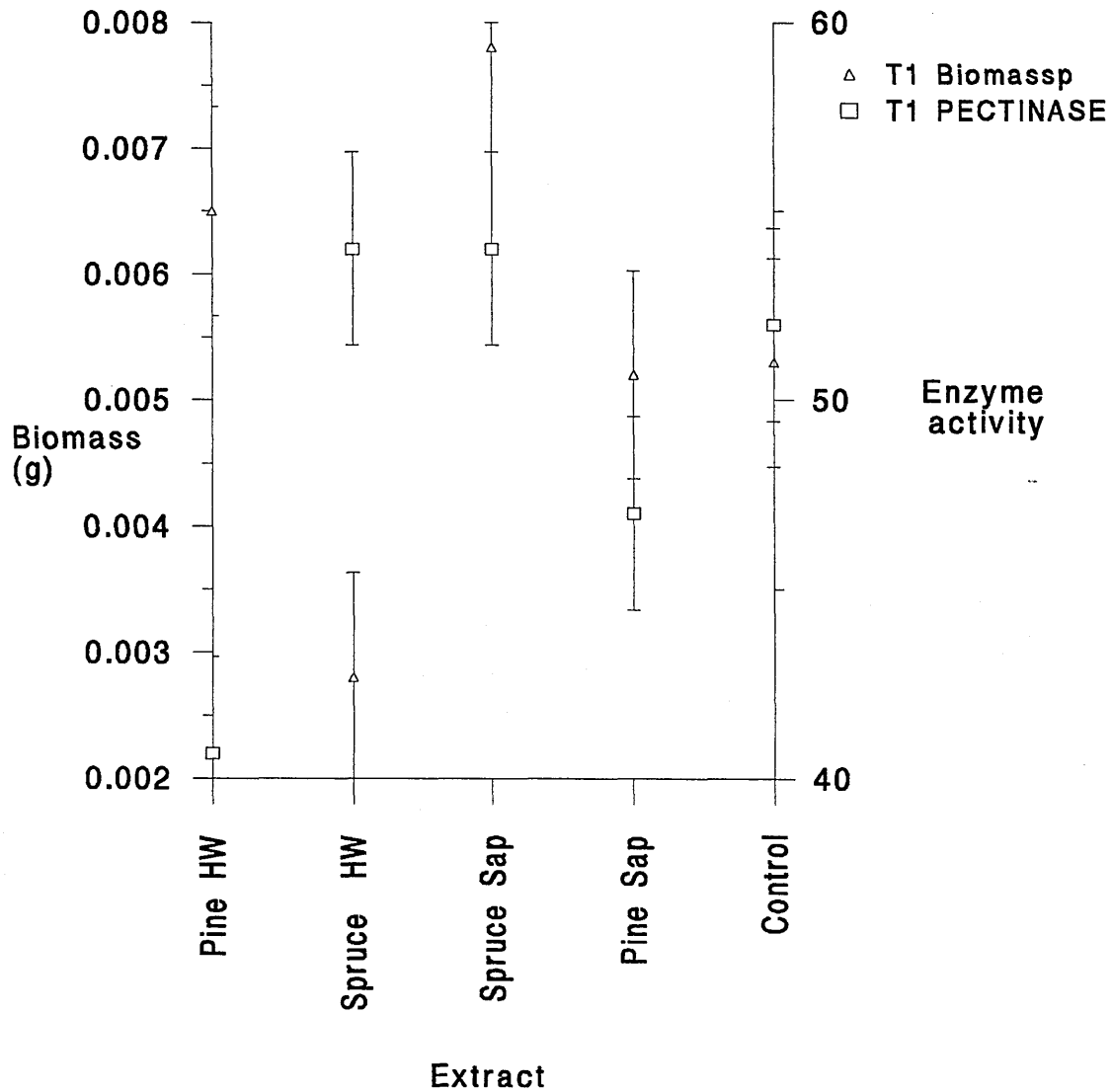


Figure 6.17 Pectinase activity of *Trichoderma viride* SIWT 70 (T35) and biomass from low nutrient medium containing pectin and wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The results shown above indicate that the biomass and the pectinase production by *Trichoderma viride* are closely linked and that generally the higher the biomass the greater the enzyme production. Pectinase activity was lowest in the filtrates from pine heartwood extracts, probably because the growth of the organism was inhibited. The two isolates

display different growth patterns and enzyme activities. *Trichoderma aureoviride* SIWT 1 shows the greatest pectinase activity between the two isolates on the spruce heartwood but has lower activity on all other medium.

6.6.3.2. Cellulase Activity

The biomass and cellulase activity of *Trichoderma aureoviride* SIWT1 (T1) when grown on low nutrient medium containing 0.5% CMC and various types of wood extract can be seen in figure 6.18.

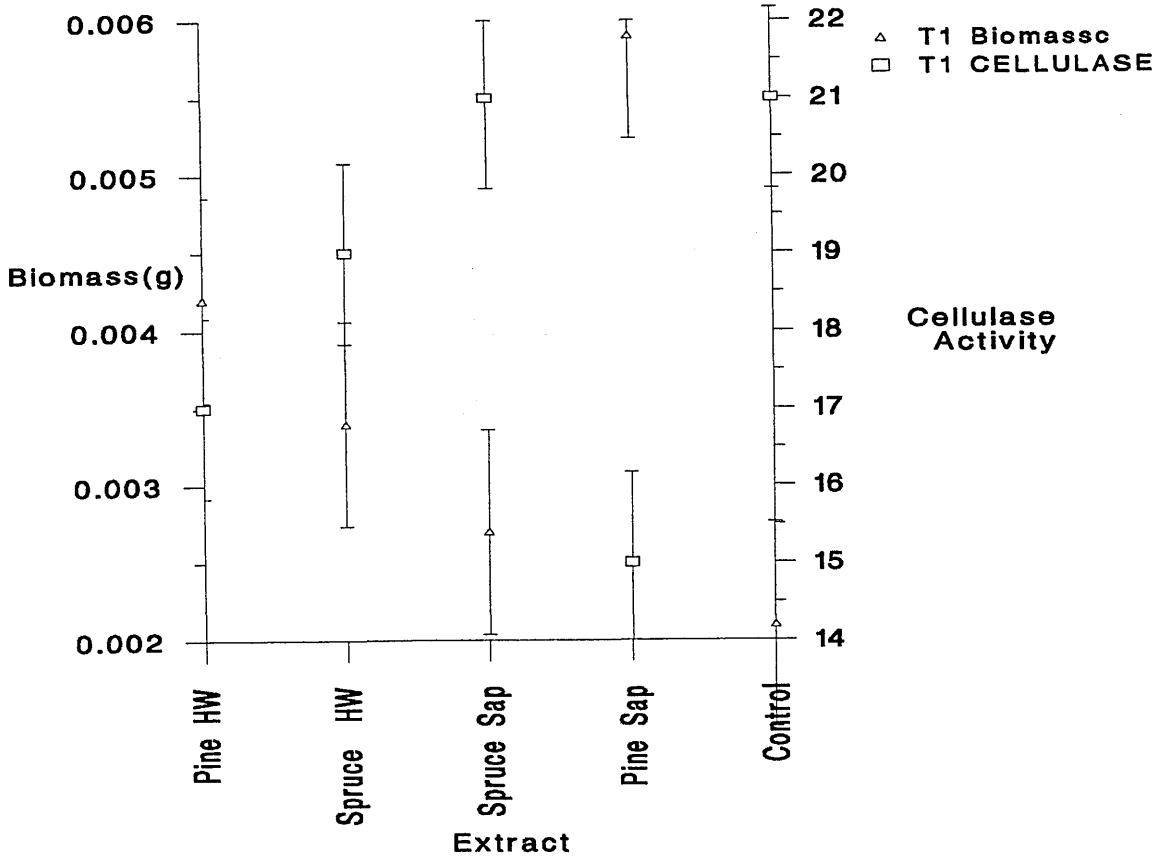


Figure 6.18 Cellulase activity of *Trichoderma aureoviride* SIWT 1 (T1) and biomass from low nutrient medium containing CMC and wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The results in figure 6.18 again show that enzyme activity in the pine extract samples is lower than in the spruce material, this may be due to the presence of sugars in the wood

extracts repressing the production of these enzymes (catabolite repression). With the spruce and control material the biomass is lower than in the pine samples but the enzyme activity is higher indicating that the organisms are utilising the cellulose in the medium to grow.

The biomass and cellulase activity of *Trichoderma viride* SIWT 70 (T35) when grown on low nutrient medium containing 0.5% CMC and various types of wood extract can be seen in figure 6.19.

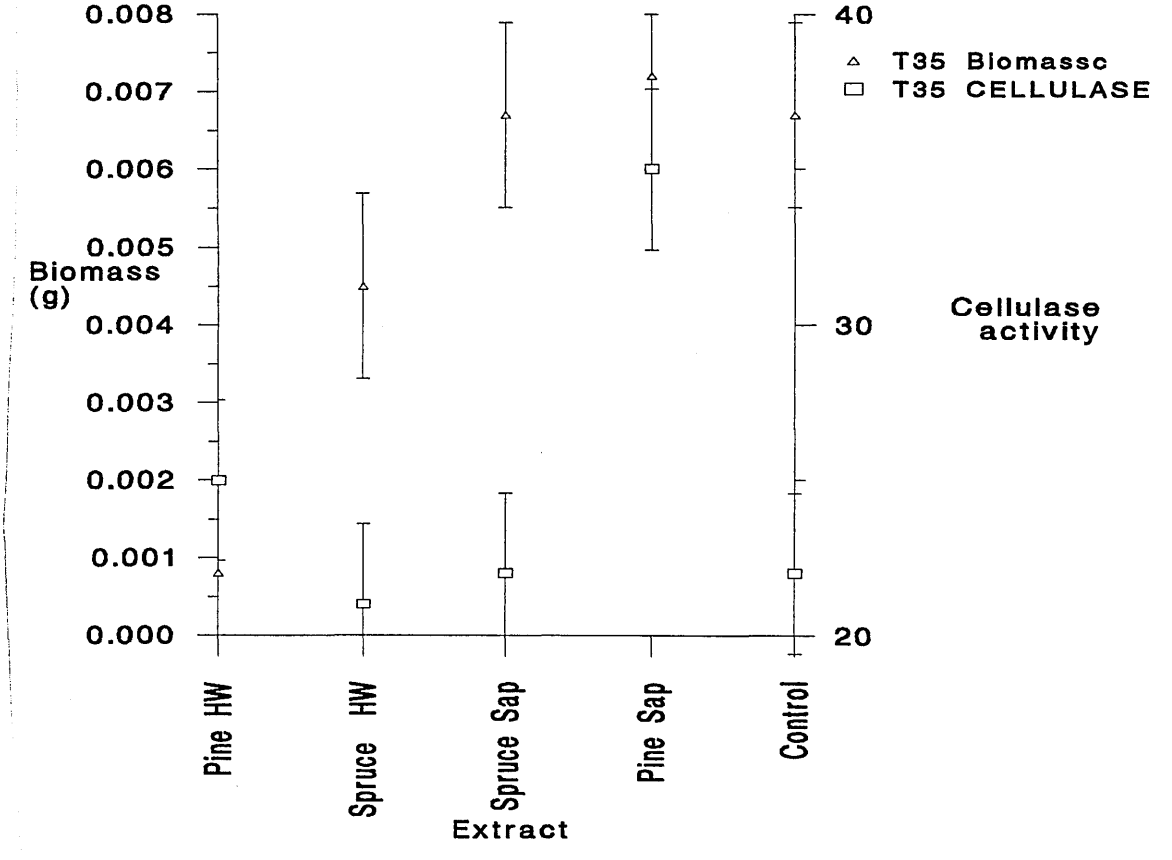


Figure 6.19 Cellulase activity of *Trichoderma viride* SIWT 70 (T35) and biomass from low nutrient medium containing CMC and wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The results in figure 6.19 show that enzyme production in the control and spruce extracts is similar to that seen with *T. aureoviride* but the biomass produced is larger. This would indicate that this organism is more efficient under these conditions at breaking down the CMC and using the products for growth. However with the pine heartwood material the organism shows a high inhibition of growth, but higher than background levels of enzyme activity indicating that the enzymes are being produced and growth is still being inhibited.

6.6.3.4 Amylase activity

The biomass and amylase activity of *Trichoderma aureoviride* SIWT 1 (T1) isolates when grown on low nutrient medium containing 0.5% soluble starch and various types of wood extract can be seen in figure 6.20.

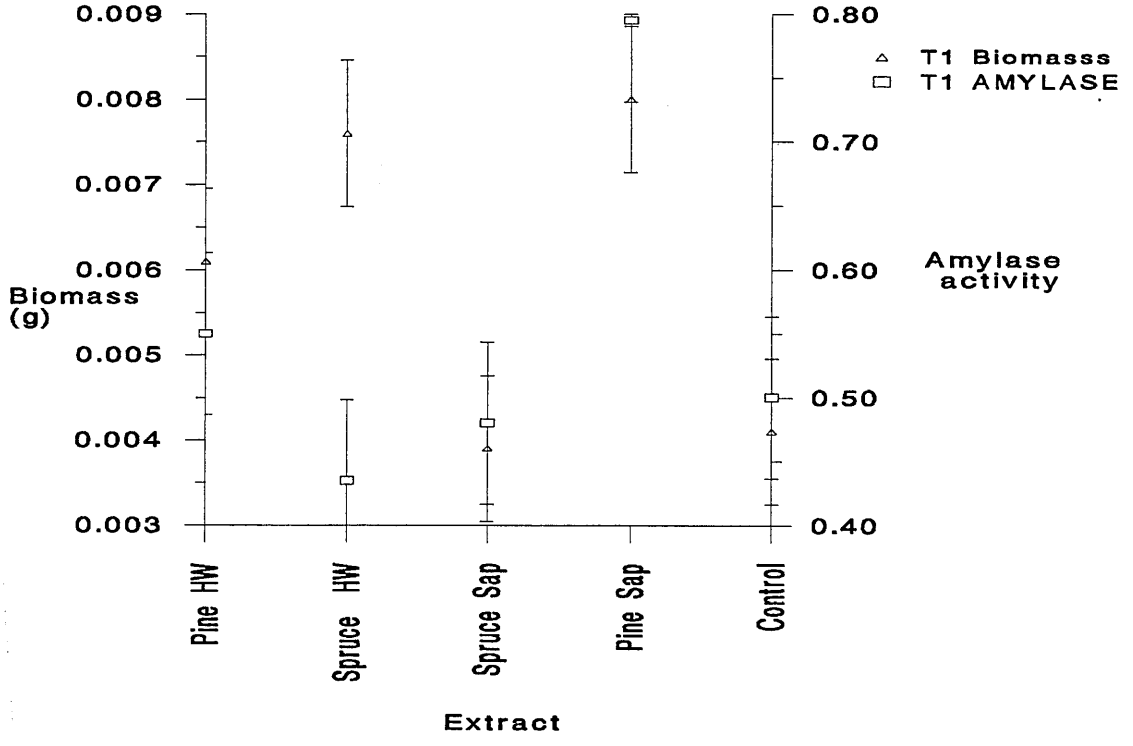


Figure 6.20 Amylase activity of *Trichoderma aureoviride* SIWT 1 (T1) and biomass from low nutrient medium containing starch and wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The results in figure 6.20 show biomass production in both pine and spruce heartwood extracts is greater than in the spruce sapwood and control extracts. The amylase activity in the spruce sapwood, heartwood and the pine heartwood samples shows similar levels to the control. The pine sapwood material however shows high amylase activity which may be due to starches being dissolved during the soxhlet extraction process supplementing the growth medium or may be due to the increased biomass being produced through mainly starch breakdown.

The biomass and amylase activity of *Trichoderma viride* SIWT 70 (T35) when grown on low nutrient medium containing 0.5% starch and various types of wood extract can be seen in figure 6.21.

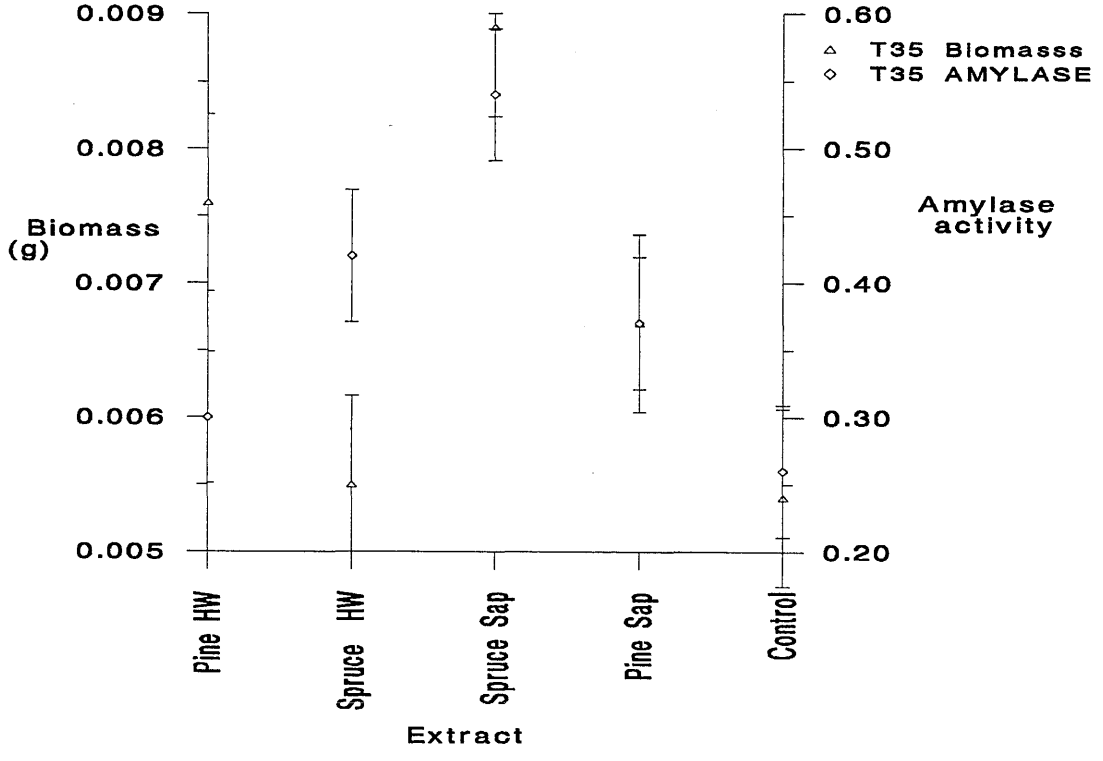


Figure 6.21 Amylase activity of *Trichoderma viride* SIWT 70 (T35) and biomass from low nutrient medium containing starch and wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The results in figure 6.21 generally indicate that the biomass production from all wood extracts was better than in the control material except spruce heartwood, this may be linked to the presence of sugars in the wood extracts that supplement the medium. The enzyme production was greatest in the spruce sapwood which also gave rise to the highest biomass. All of the extracts gave higher amylase production than in the control material, which may be due to induction of enzymes due to low levels of product sugars.

6.6.3.4 Reducing sugar assay

The concentration of reducing sugars in the wood extracts can be seen in figure 6.22

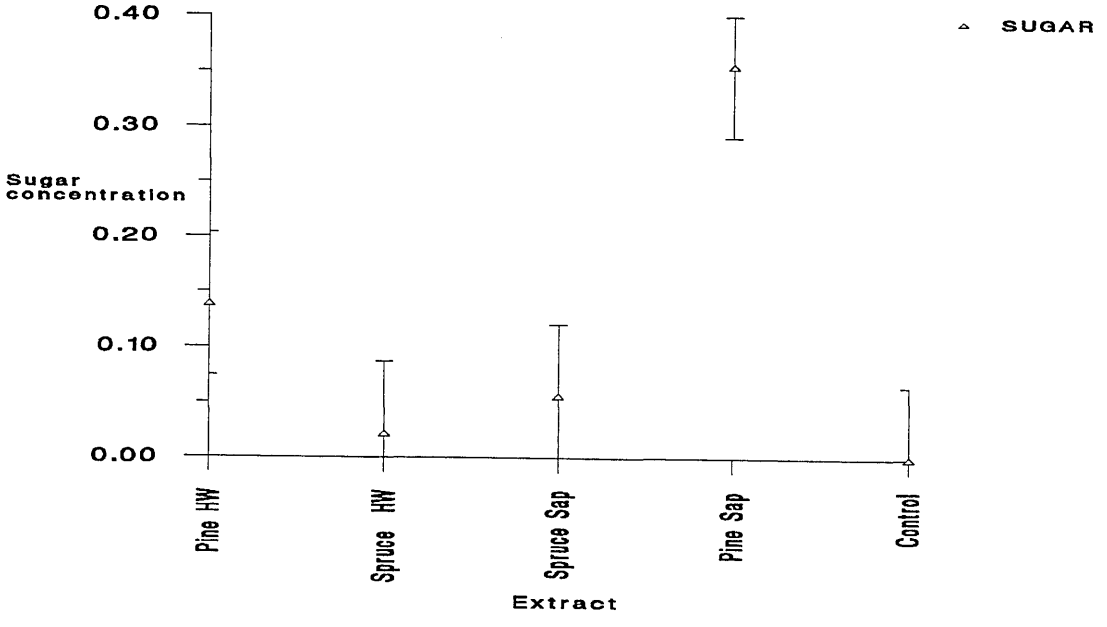


Figure 6.22 Sugar concentrations (mg/ml) of hot water wood extracts. N.B. Error bars were calculated as the standard error of the data set.

The above results show that both the pine extracts have significant concentrations of reducing sugars that will supplement the growth medium and may have a significant effect as inducers/ repressors of any enzymes produced by the *Trichoderma* isolates.

6.6.5. Discussion.

Growth of *Trichoderma spp.* is dependent on the utilisation of nutrients in the growth medium. To utilise these nutrients effectively organisms have to produce enzymes to breakdown the complex carbohydrates into simple compounds. The production of extracellular enzymes by *Trichoderma* spp. may be induced by the presence of different compounds in the growth substrate (Priest, 1984).

Complex growth substrates will induce fungi to produce a range of different enzymes. The production of induced enzymes can be altered by the presence of different carbon sources while the production of certain enzymes may also be repressed if product sugars are present (catabolite repression).

From these experiments it is difficult to differentiate between the productivity and activity of enzymes produced by the organisms. It is possible that the organisms may be producing similar quantities of the enzymes but because of inhibitory compounds in the medium the enzymes are unable to act effectively. Hence all of the results are based on the activity of the enzymes. It can be seen that the effects of some of the different extracts are to induce and repress the activity of some of the target enzymes.

The growth of the *Trichoderma* isolates appears to vary between the different carbon sources. With most of the wood extracts the growth was better than that recorded in the controls. This may be due to the extra nutrients in the wood extracts that supplement the low nutrient medium giving rise to better growth as well as having an effect on the production/ activity of the enzymes. The two *Trichoderma* isolates appear to differ with respect to the amount of biomass produced over the incubation period. In previous experiments the organisms grown on pine heartwood extracts showed the lowest growth, this did not always appear to be the case in this experiment. The difference here is that

pectin, cellulose or starch is used as the sole carbon source that is available for growth to the organisms.

There is no direct correlation between enzyme production by the *Trichoderma* isolates and biomass production by the organisms, this would indicate the biomass being produced is not be linked solely to the enzyme activity. With the pectinase activity for *T. aureoviride* (figure 6.16) the pine heartwood material produced a high weight gain after incubation but the enzyme activity was lower than for the other extractives. This may be caused by the organism growing on the supplementary nutrient in the added wood extract or the organism is producing the enzyme at the same levels as in the other extracts but the activity of the enzyme is being reduced by the presence of the extracts. In other instances higher enzyme activity was recorded in samples which produced lower biomass. This is likely to be a response by the organism where high levels of the enzyme are produced to break down the substrate but the wood extract inhibited the growth of the organism.

The breakdown of complex substances like cellulose and pectin are linked to a number of enzymes which will work in sequence on the substrate breaking it down to small products, an example of this can be seen in figure 6.23.

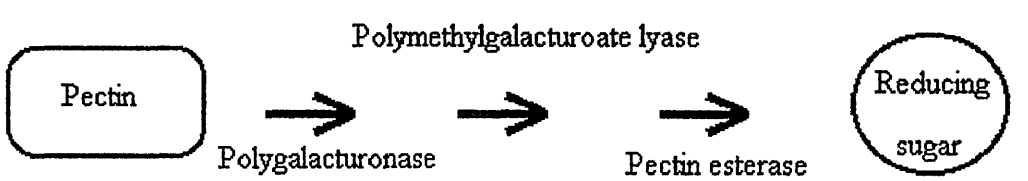


Figure 6.23 Enzyme sequence for the breakdown of Pectin

From figure 6.23 it is possible to appreciate that the action of any of these enzymes would result in a loss in viscosity of a pectin solution. If the path was blocked by the presence of inhibitory compounds it is possible that the viscosity of the pectin solution would still be reduced although the complete breakdown of the substrate may not be achieved and hence increases in biomass by the utilisation of this material would not result. This would give rise to the result where high activity was reported but little biomass was produced e.g. figure 6.17 Sitka spruce heartwood sample. Similar results for cellulase could be explained in this manner as the breakdown of cellulose is also a multi-enzyme pathway.

With the introduction of wood extracts into the medium, several extra factors can affect the growth and enzyme production of the isolates. The wood extracts used have previously been shown to generally reduce the biomass produced but this was not always the case with the target carbon sources used in this experiment where the organisms grown on the medium containing the extract and carbon source showed better growth. Importantly the wood extracts contained sugars which were dissolved during extraction, these supplemented the medium and permitted better growth than was experienced in the control material (figure 6.18 Sitka spruce sapwood extract). The introduction of these sugars also created the possibility of catabolite repression, where the sugars were utilised for growth before the target enzymes were produced (figure 6.19, Scots pine sapwood).

Overall the *Trichoderma* isolates grown on the wood extracts in all cases produced measurable amounts of the enzymes and in all cases there was sufficient enzyme present to cause a reaction in the cellulase, pectinase and amylase assays. This would imply that if the organisms were to grow in wood in the presence of the compounds found in these extracts then they would still be capable of improving the permeability of the timber.

Chapter 7. Effect of *Trichoderma* on the permeability of fresh Scots pine and Sitka spruce logs.

7.1 Introduction

From earlier experiments (Chapter 4) it can be seen that selected *Trichoderma* isolates can improve the permeability of both Scots pine and Sitka spruce blocks. The growth of fungi through timber has been shown to improve its subsequent permeability, but the physical condition of the timber has long been known to limit the growth of organisms within (Hof, 1981). In particular the moisture content has been shown to be a major limiting factor (Nicholas and Siau,1973) with timber that is too wet being incapable of supporting growth of selected organisms and timber that has dried to below fibre saturation point has also been shown to be incapable of supporting fungal growth. The latter is the basis of using seasoned timber in non-preservative treated state and is generally sufficient for use in dry conditions out of contact with the ground.

Trichoderma are pioneer colonising organisms and if the permeabilities of different timber types are to be improved by the action of *Trichoderma* isolates it is essential that these isolates colonise the timber quickly after felling before other organisms are able to establish. Since felling and conversion of timber is generally done in a green state it is essential that selected isolates are capable of colonising timber in this state. Fluoride has been shown to act as an inhibitor of fungal growth (Lindgren and Harvey, 1952) and at lower concentrations has been seen to encourage the growth of *Trichoderma* isolates. Lindgren and Harvey (1952) noted that logs sprayed with 2% sodium fluoride were colonised by *Trichoderma* isolates.

The aim of this experiment was to determine the effectiveness of colonising roundwood logs with *Trichoderma* isolates and subsequent effects on the permeability of freshly felled Scots pine and Sitka spruce logs and hence establishing the most appropriate methodology for delivering *Trichoderma* into larger poles.

7.2 Methods

Scots pine and Sitka spruce trees up to 12 inches in diameter were felled at a site near Kirriemuir (Tayside, Scotland). The timber was then sectioned into 50cm long roundwood logs. Logs were marked to identify from which tree each log had come and end sealed with a fast drying varnish (Ronseal solvent free varnish, Ronseal UK LTD). Sealing was carried out in the forest in order to minimise contamination and moisture loss from the logs. The logs were then transported to the University for further treatment.

A total of 96 logs were processed using 4 different treatment regimes (12 pine and 12 spruce logs per treatment).

Bark is the first line of defence of trees to infection. It also prevents desiccation and if pole material is to be treated in the forest it would be preferable to treat it with the bark intact. One group of 24 logs (12 pine and 12 spruce) were therefore left barked for subsequent inoculation with pellets containing the selected *Trichoderma* isolates. The remaining logs were debarked prior to treatment.

Twenty four of the debarked logs were sprayed with 2% sodium fluoride to encourage subsequent growth of selected *Trichoderma* isolates, while 24 other logs were sprayed with 8% fluoride to prevent any surface colonisation by contaminant organisms through the debarked surfaces. The final 24 debarked logs were treated solely with the *Trichoderma*.

For each timber species four replicate logs from separate trees were prepared for each treatment prior to inoculation with either of the two *Trichoderma* isolates. Control logs were also prepared using the treatment regimes in Table 7.1 but the logs were not inoculated with either of the *Trichoderma* isolates.

Barked / Debarked	Fluoride spray 0%, 2% or 8%	Pellet or spore inoculation
1) Barked	0%	Pellet
2)Debarked	0%	Spore
3)Debarked	2%	Spore
4) Debarked	8%	Pellet

Table 7.1 Treatments of Scots pine and Sitka spruce using different delivery systems for the two *Trichoderma* isolates.

Prior to *Trichoderma* treatment all logs were cored using a plug cutter (internal diameter 12.5 mm). Logs were bolted in position and drilled using a medium speed pillar drill. Cores were removed from 3 positions on each log (Figure 7.1). This provided reference cores from which changes in permeability could be assessed after incubation. Cores were removed asceptically from the logs and frozen for future analysis (as described in Chapter 5). Holes left by the removal of cores were then plugged with sterile Scots pine dowel . Plate 7.1 shows a pine log after sampling.

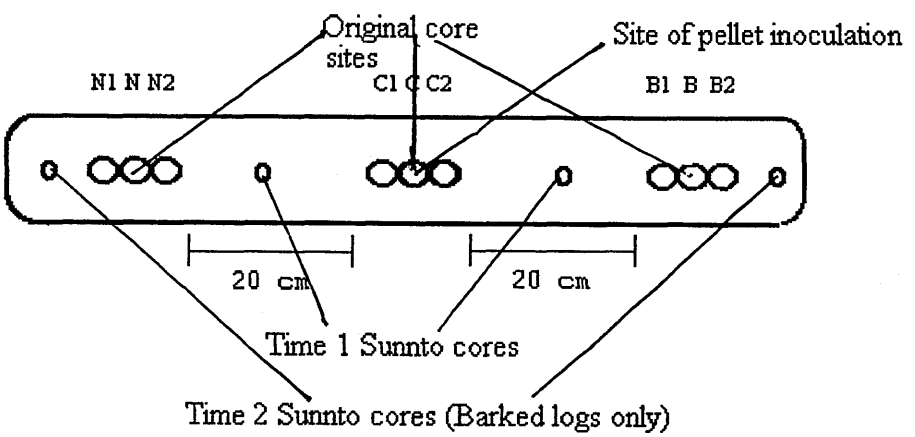


Figure 7.1 Diagram showing positions of cores removed from sample log sections. (Positions N, C and B represent reference cores removed prior to *Trichoderma* inoculation while $N_{1,}N_{2,}$, $B_{1,}B_{2,}$, and $C_{1,}C_{2,}$ represent cores removed after incubation was complete to determine subsequent changes in permeability. Time 1 and 2 cores for *Trichoderma* establishment represent 12 & 16 weeks after inoculation respectively

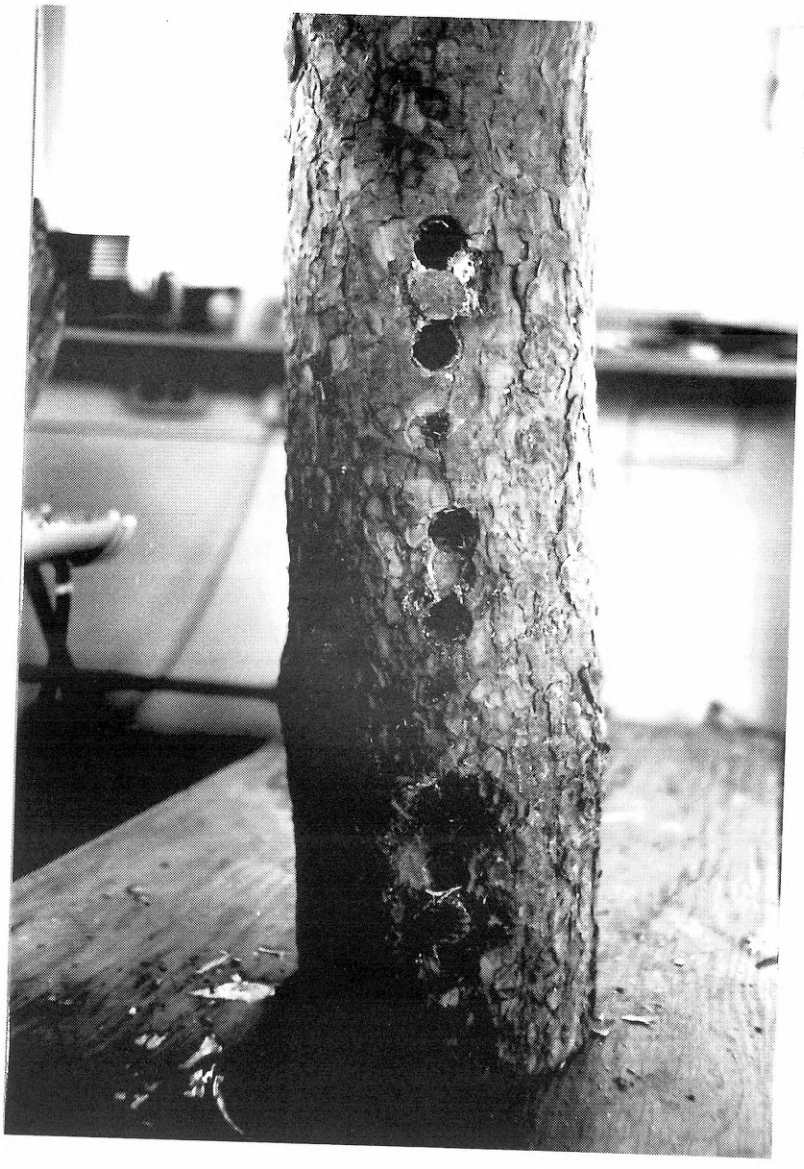


Plate 7.1: Pine log showing sampling points for removal of cores for permeability measurements.

In order to inoculate the logs with the selected *Trichoderma* isolates suitable delivery systems were required. Two methods were used in this experiment:

1) Material that was debarked and treated with 0 or 2% fluoride was inoculated with a spore suspension sprayed onto the surface of the logs (treatments 2 & 3 Table 7.1). Spore suspensions were prepared by pouring sterile water onto plates of sporulating *Trichoderma* isolates and agitating the surface of the colony to release the spores into the liquid. The liquid was then transferred into a sterile beaker and the process repeated until sufficient spore suspension was collected. The concentrations of the spores in the suspension was then determined using a haemocytometer. Twenty five millilitres of a 6 x 10^6 spores/ ml concentration of *Trichoderma* isolates was applied to each log.

2) Material that was left barked or debarked and treated with 8% fluoride was inoculated with pellets of the selected *Trichoderma* material to examine whether this different treatment regime would give better permeability enhancement through more even colonisation. While bark would limit colonisation by contaminant organisms on debarked logs, the 8% Fluoride would limit surface colonisation by contaminants while allowing drying of the logs similar to that in spore treated logs.

Pellets were prepared following the method described by Budge and Whips (1981) where *Trichoderma* isolates were grown on a mixture of bran flakes and perlite. Bran flakes were ground to a fine powder and 200mls placed in a glass jar, 15% (v/v) perlite was added and mixed throughout the bran flakes, 50 ml of distilled water was then added and the mixture autoclaved at 121^{o}C for 20 minutes. The jars were then inoculated with either of the selected *Trichoderma* isolates and incubated for 2 weeks at the optimum temperature (22 or 25^{o}C) for each isolate. After incubation 50 mls of sterile water was added to the pellet mixture. The activity of the mixture was then confirmed by plating out samples of the material onto 3% malt extract agar plates. Having established the viability of the *Trichoderma* spores in the pellet mixture, the mix was then transferred into sterile

pressure tubes from which the pellets were extruded into the logs. Each log was inoculated with 2.5 cm of pellet material being deposited in the central core site (C) on the log (see figure 7.1).

After *Trichoderma* treatment all logs were placed inside two autoclave bags to reduce the rate of drying and raise the humidity around the logs in order to aid colonisation and growth of the isolates through the timber. All logs were incubated at ambient temperature for a total of 26 weeks. Control logs, uninoculated with *Trichoderma* isolates were incubated at a separate location to avoid cross contamination by the selected *Trichoderma* isolates.

The establishment of *Trichoderma* isolates was monitored by the removal of cores from the logs at set locations after 12 weeks for all logs and 16 weeks for barked logs (see figure 7.1). Cores were sectioned into sapwood and heartwood material before being plated out onto 3 % malt extract agar and incubated at 22 or 25^{o}C. The plates were then monitored daily for any subsequent growth.

After 26 weeks six cores were removed from each log at sites adjacent to original permeability core sites (see figure 7.1 sites $N_{1,}$,$N_{2,}$, $B_{1,}$,$B_{2,}$, and $C_{1,}$,C_{2}).
The air permeability in a radial direction was measured on all cores removed before inoculation and samples removed from the adjacent sites after incubation with the *Trichoderma* isolates. The method used to measure air permeability was that described in Chapter 5. Corresponding cores (e.g. $N_{1,}$, N and $N_{2,}$) were placed side by side and cut into equal lengths (approximately 1.5 cm sections) for moisture content and air permeability analysis (see figure 7.2).

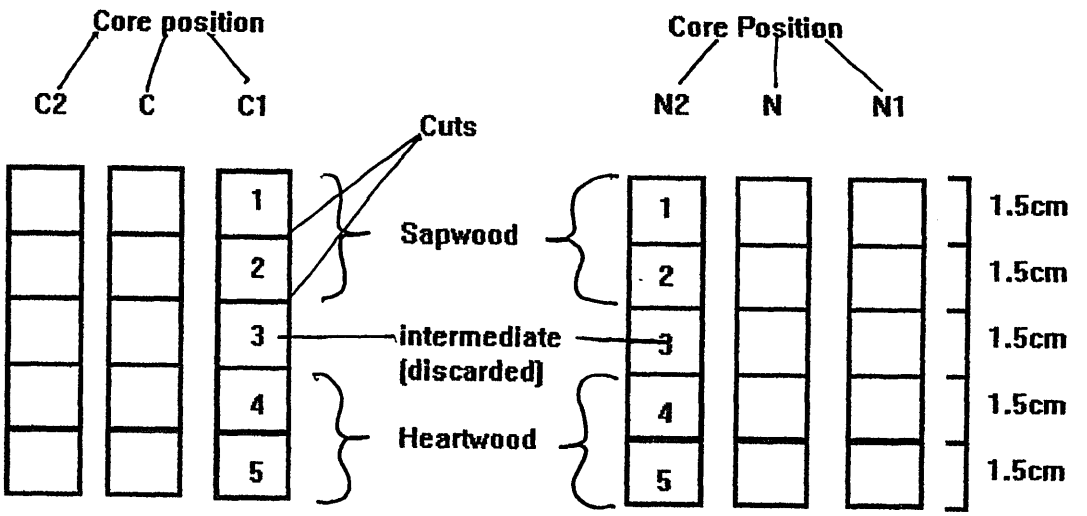


Figure 7.2 Diagram of sectioning of core samples removed from adjacent sites of the log.

The permeabilities of sections removed from corresponding sites on adjacent cores were compared and the % increase in permeability calculated as follows:

((permeability of adjacent core after incubation $N_{1,}N_{2,}$ $B_{1,}B_{2,}$ $C_{1,}C_{2}$)- (Permeability of reference cores N, B, C)) / (Permeability of reference cores N, B, C) x 100

7.3 Results.

The results in Table 7.2a show that *Trichoderma* isolates had successfully colonised the sapwood of the logs. The smaller number of isolations from the ends of barked logs indicate that colonisation from the pellet inoculum may be slower. The control logs also showed some *Trichoderma* growth as well as other mould species. Since this system is a non-sterile one it is reasonable that other organisms may become established on the logs.

Timber	Treatment group	*Trichoderma aureoviride*	*Trichoderma viride*	Control
Scots pine	Barked (mid)	81%	81%	38%
	Barked (end)	88%	38%	19%
	Debarked	75%	56%	40%
	2%NaF	88%	81%	69%
	8%NaF	75%	44%	29%
Sitka spruce	Barked (mid)	75%	75%	6%
	Barked (end)	13%	38%	0%
	Debarked	81%	81%	31%
	2%NaF	75%	63%	44%
	8%NaF	88%	69%	25%

Table 7.2a. Isolation of *Trichoderma* isolates from the Sapwood of Scots pine and Sitka spruce logs after 12 or 16 weeks incubation. (N.B. Barked end samples represent isolations from end log locations after 16 weeks incubation). NB % was calculated from the number of observations of the *Trichoderma* / number of cores removed from the log x 100.

It is noticeable that controls treated with 2% Fluoride showed higher *Trichoderma* colonisation than other control groups and surprisingly 8% fluoride treated also gave significant reisolation of *Trichoderma* isolates.

Results in Table 7.2b indicate that *Trichoderma* has become established in the heartwood region of the timber. Although the fact that the isolate grew from samples does not necessarily mean that the permeability of this region would also be increased.

Timber	Treatment	*Trichoderma aureoviride*	*Trichoderma viride*	Control
Scots pine	Barked	56%	19%	13%
	Debarked	75%	25%	40%
	2%NaF	88%	50%	50%
	8%NaF	81%	88%	25%
Sitka spruce	Barked	83%	46%	0%
	Debarked	94%	88%	31%
	2%NaF	69%	63%	56%
	8%NaF	100%	19%	56%

Table 7.2b. Isolation of *Trichoderma* isolates from the Heartwood of Scots pine and Sitka spruce logs after 12 weeks incubation. NB % was calculated from the number of observations of the *Trichoderma* / number of cores removed from the log x 100.

The spread of *Trichoderma aureoviride* throughout the heartwood is similar to that seen in the sapwood for both Scots pine and Sitka spruce material. However it is clear that the reisolation of *Trichoderma viride* is lower in the heartwood regions with the exception of the 8% fluoride Scots pine samples.

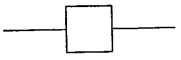
Air permeability was measured on cores removed from adjacent sites on the log and the results of these determinations are shown in figures 7.3-7.18.

These figures represent the % increase in permeability at different depths from cores removed from Scots pine and Sitka spruce logs after incubation with *Trichoderma* isolates. For each log a total of 9 cores were removed, 3 reference cores prior to incubation and 6 cores from adjacent sites after the completion of the incubation.

The results shown in figures 7.3-7.18 indicate the number (frequency) of individual cores which after *Trichoderma* incubation produce various % permeability increases. Since no significant increases in permeability were noted between the sapwood samples from different depths the results for the inner and outer sapwood regions were combined. As 4 logs were used for each treatment the maximum number of observations for sapwood was 48 and 24 observation sites for heartwood.

Key to figures 7.3- 7.18

Cores Inoculated with *Trichoderma aureoviride* SIWT 1



Cores Inoculated with *Trichoderma viride* SIWT 70

Cores removed from control material.

Scots pine Logs.

The frequency of increased permeability of sapwood material from Scots pine logs treated with 2% fluoride and inoculated with selected *Trichoderma* can be seen in figure 7.3.

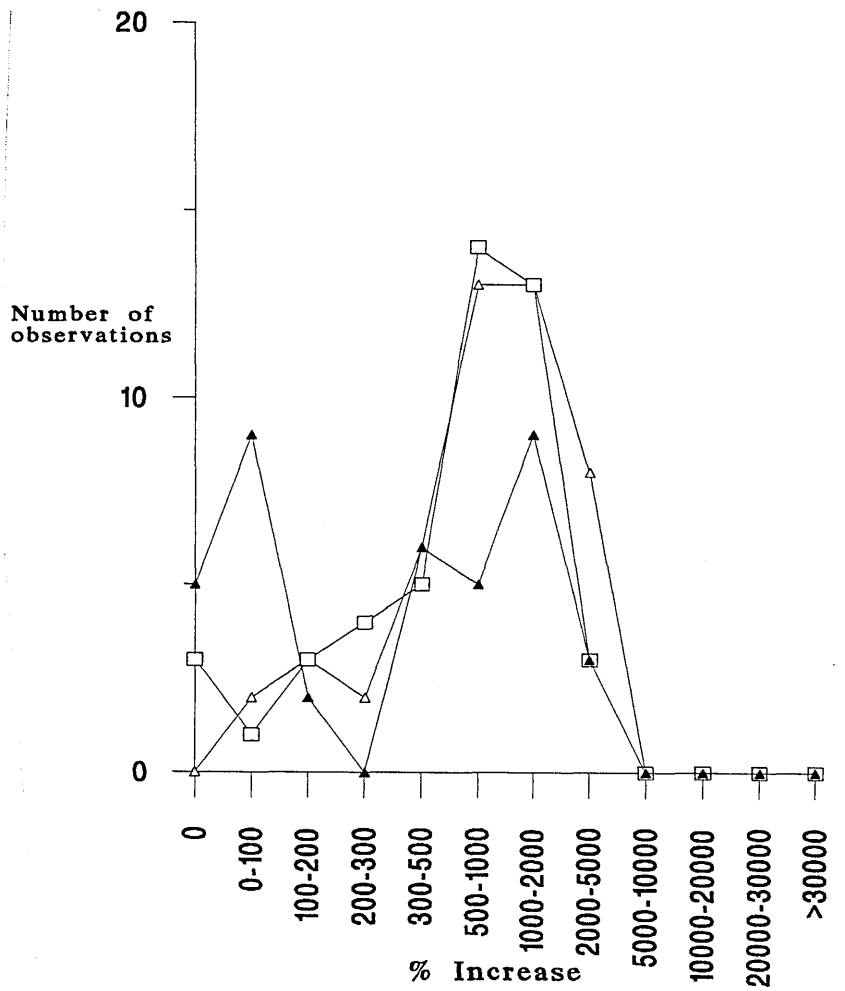


Figure 7.3 Frequency (number of core sections giving an increase in permeability in Scots pine sapwood) of % increases in permeability of Scots pine logs treated with 2% fluoride and selected *Trichoderma* isolates (*Trichoderma aureoviride* SIWT 1 and *Trichoderma viride* SIWT 70).

This figure shows that in the majority of samples the permeability after *Trichoderma* and control incubation is increased in the sapwood. When these samples were analysed using Analysis of Variance the *Trichoderma* treated samples however showed no significant increases in permeability over the control material (within 95% confidence limits).

The analysis of the pine samples showed that there may have been increases in the permeability of the sapwood samples, however at 90% confidence limits the pine sapwood samples treated with *Trichoderma aureoviride* showed a significant increase in permeability compared to control material. This may indicate that this isolate has had an effect on the permeability of the timber, further investigations would be required to confirm this.

The results of the permeability enhancement in Scots pine heartwood treated with selected *Trichoderma* isolates can be seen in figure 7.4.

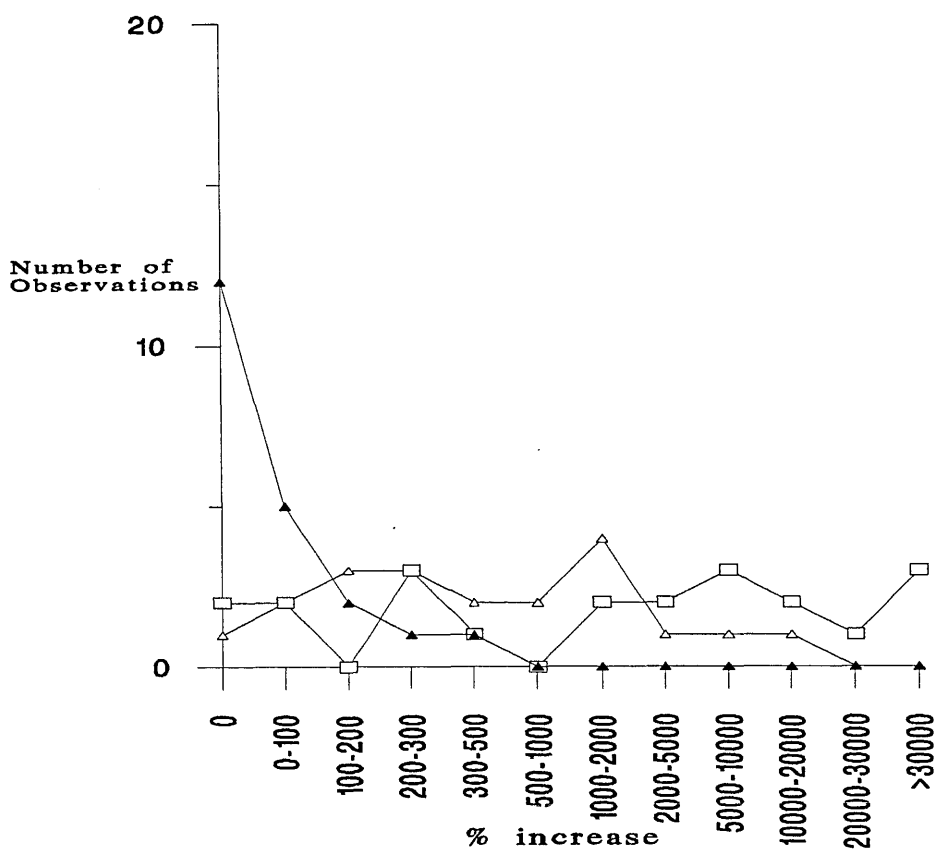


Figure 7.4 Frequency (number of core sections giving an increase in permeability in Scots pine heartwood) of % increases in permeability of Scots pine logs treated with 2% fluoride and selected *Trichoderma* isolates (*Trichoderma aureoviride* SIWT 1 and *Trichoderma viride* SIWT 70).

Heartwood core sections removed from the *Trichoderma* treated pine logs showed a statistically significant increase in permeability when compared to control samples, both isolates gave a significant increase in permeability after incubation.

Pine control logs treated with 2% Fluoride show generally lower % increases in permeability, this may be due to other *Trichoderma* isolates being able to colonise the timber but being less effective in their ability to increase the permeability of the pine material.

Figure 7.3 shows the % increases in the sapwood permeability but does not represent the actual permeabilities observed in the samples. Representative values for the permeabilities at each depth can be seen in Table 7.3.

Trichoderma isolate	Core position (see figure 6.1)	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	843	1182	19.1
	N	166	146	5.3
	N2	1512	1135	20.2
	C1	2227	1172	35.5
	C	103	169	17.6
	C2	2686	1473	20.1
	B1	2420	869170	57.2
	B	170	1199	3.3
	B2	2052		25.3
T.viride	N1	1605	1558	1.4
	N	200	146	0.2
	N2	2269	1292	2.5
	C1	1314	1555	20.1
	C	111	94	5.4
	C2	1327	2082	17.4
	B1	971	1882	22.2
	B	151	108	0.7
	B2	1766	1189	17.1

Table 7.3 Representative values for actual permeability measurements from cores removed from Scots pine logs treated with 2% fluoride and *Trichoderma* isolates.

The results in table 7.3 show that the cores removed from sites adjacent to the original core site have a larger permeability after *Trichoderma* treatment and highlight the extent to which heartwood material is less permeable than the sapwood even after *Trichoderma* treatment.

The frequency of increased permeability in the sapwood of debarked Scots pine logs treated with 8% fluoride and inoculated with pellets of the selected *Trichoderma* isolates can be seen in figure 7.5.

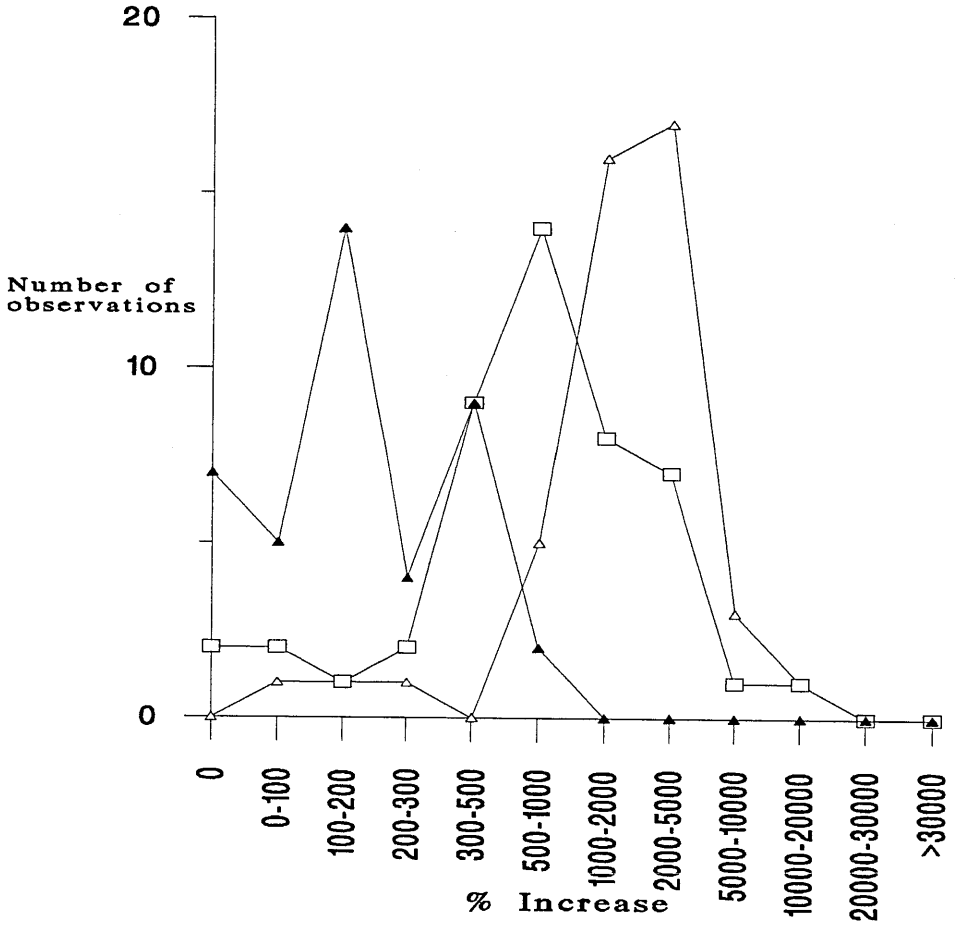


Figure 7.5 Frequency (number of core sections giving an increase in permeability in Scots pine sapwood) of % increases in permeability of Scots pine sapwood treated with 8% fluoride and selected *Trichoderma* isolates.

This figure shows that in all sapwood samples the permeabilities after *Trichoderma* colonisation are increased. The distribution shows that the % increase peaks at between 2000 and 5000%. When analysed the increases in permeability after *Trichoderma* treatment are significantly greater than those observed in control material (at 95% confidence limits).

Heartwood material from the pine logs treated with 8% NaF showed the changes in permeability described in figure 7.6.

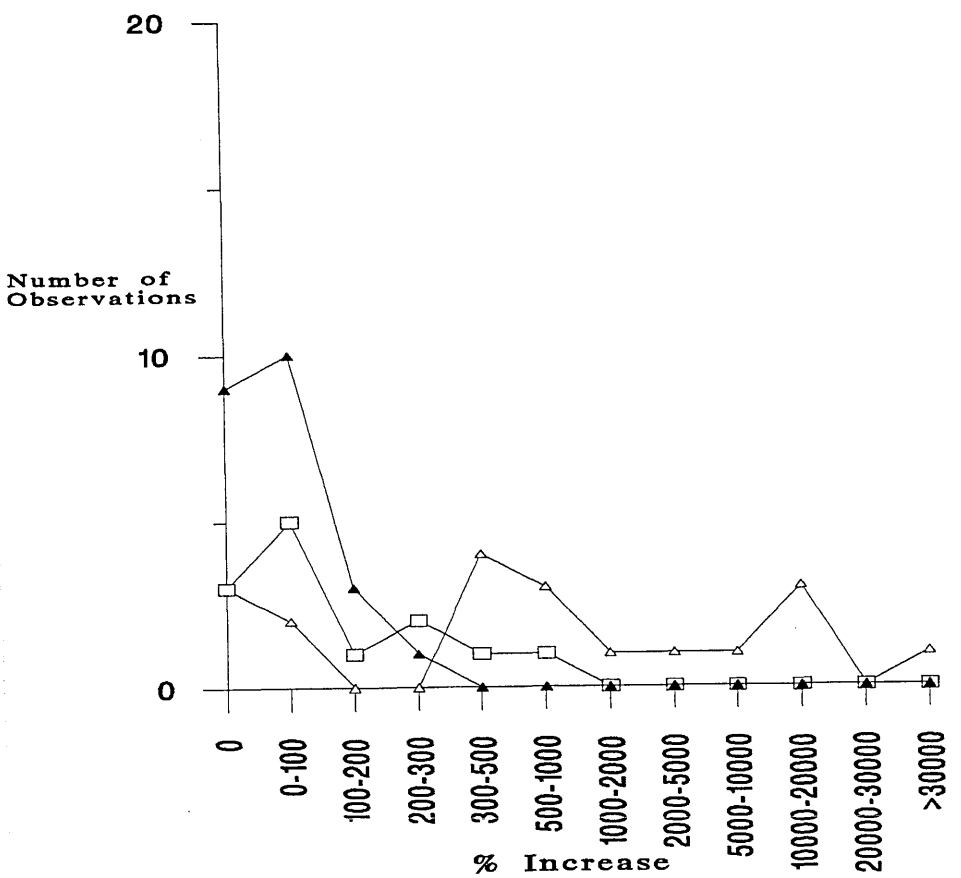


Figure 7.6 Frequency (number of core sections giving an increase in permeability in Scots pine heartwood) of % increases in permeability of Scots pine heartwood treated with 8% fluoride and selected *Trichoderma* isolates.

Heartwood material shows generally lower increases in permeability compared with the surface inoculation with *Trichoderma* on the 2% fluoride samples. However when these were compared to control material the increases in permeability after *Trichoderma* treatment were significant at 95% confidence limits.

These measurements again show only the % increases in permeability. Representative figures showing actual permeabilities can be seen in Table 7.4.

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	1235	1568	21.2
	N	55	37	4.3
	N2	1272	1130	41
	C1	1663	2862	57
	C	97	48	11.5
	C2	2179	2799	114
	B1	2331	2361	11.9
	B	53	26	11.9
	B2	3010	2810	157
T.viride	N1	817	367	14.61
	N	96	97	38
	N2	2255	1307	41
	C1	2576	1241	226
	C	59	116	18
	C2	2874	1550	110
	B1	2102	1117	83
	B	66	98	47
	B2	2187	907	22

Table 7.4 Representative values for radial air permeability from Scots pine logs treated with 8% fluoride and inoculated with *Trichoderma* isolates.

The results in table 7.4 show that there is an increase in permeability in the sapwood region after exposure to *Trichoderma* and that the actual permeability of the samples is similar to those seen in table 7.3. The increases in the heartwood region are not as marked and in some cases no increase in permeability was observed.

The frequency of increased permeability in barked Scots pine logs treated with selected *Trichoderma* isolates can be seen in figure 7.7.

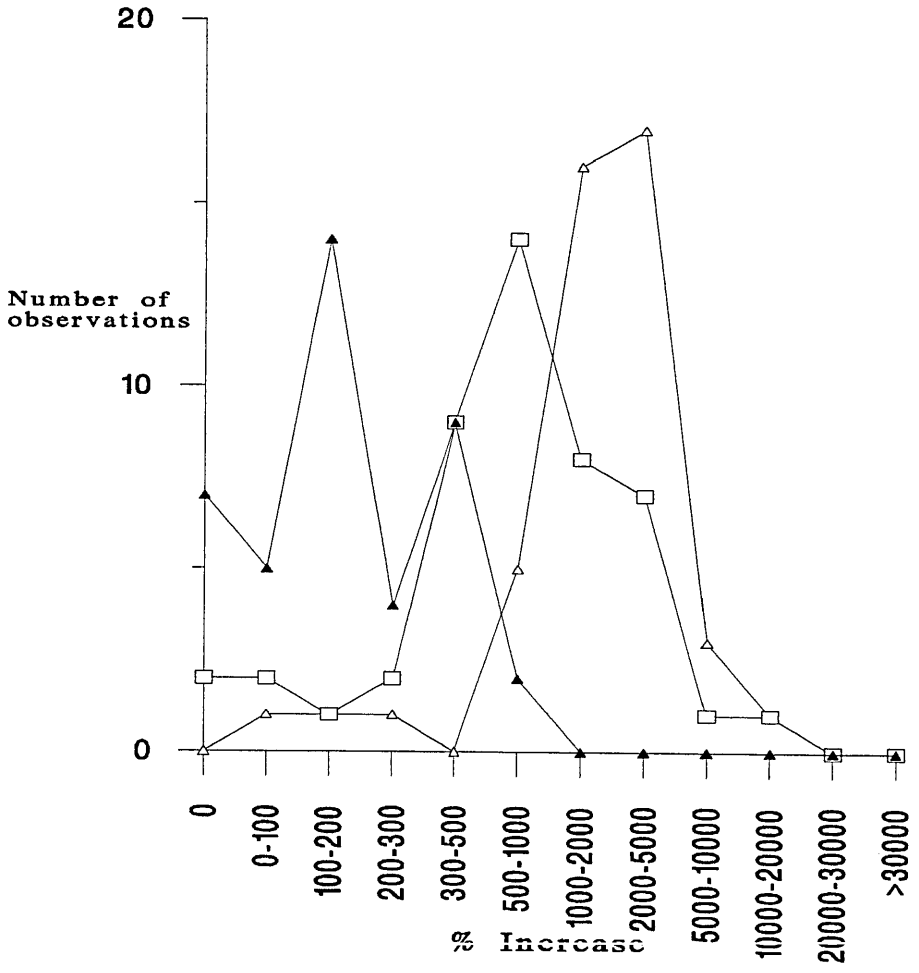
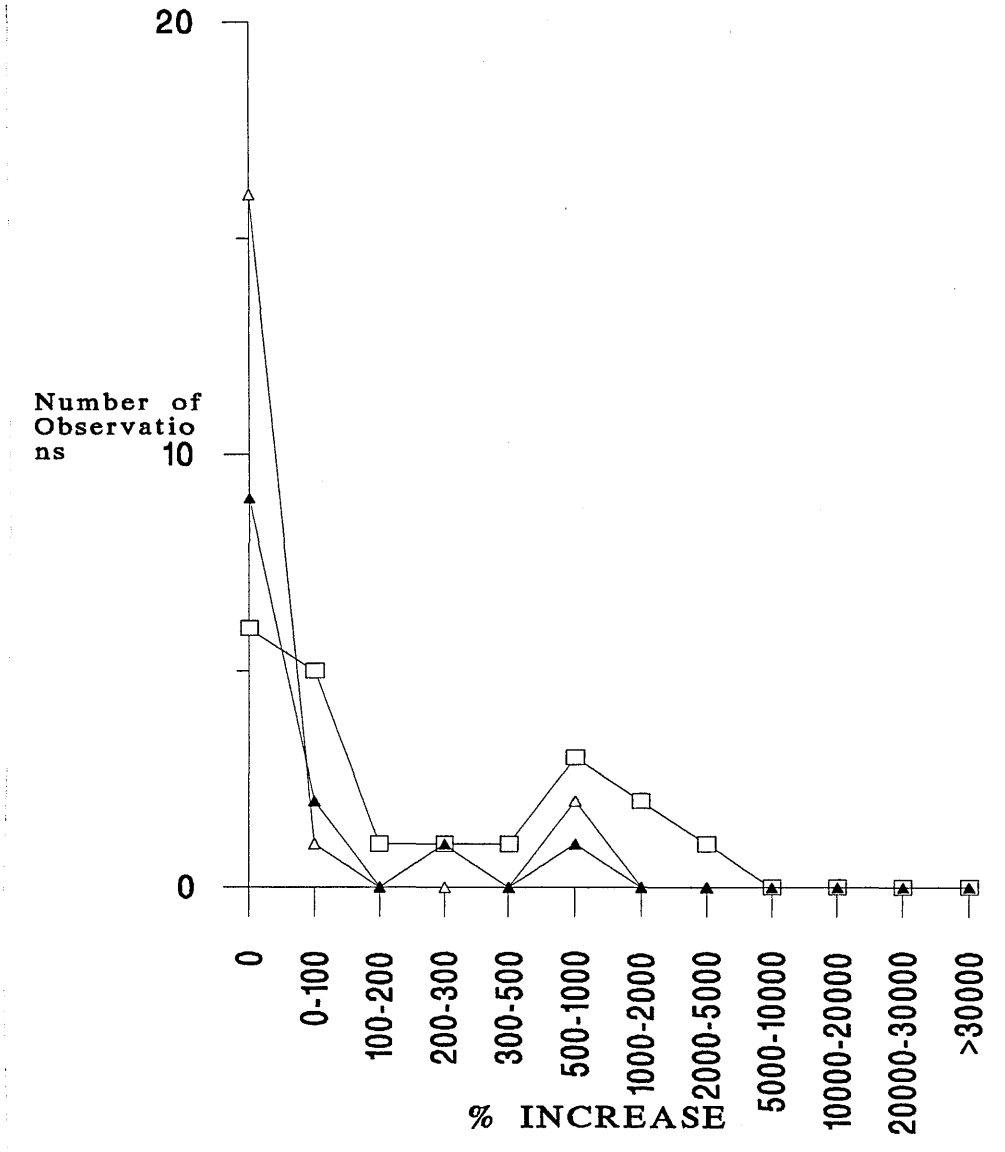


Figure 7.7 Frequency (number of core sections giving an increase in permeability in Scots pine sapwood) of % increases in permeability of barked Scots pine sapwood treated with selected *Trichoderma* isolates.

The results in figure 7.7 show that a large number of sapwood samples show an increase in permeability after treatment with the selected *Trichoderma* isolates. When analysed statistically these increases are significant at 95% confidence limits for *Trichoderma aureoviride* SIWT 1 and 90% confidence limits for *Trichoderma viride* SIWT 70. The differences between the organisms may be due to the colonisation pattern exhibited by the organism as they grew through the logs. Also the largest increases in permeability were experienced at the sites adjacent to the inoculation point. Evidence of this can be seen more clearly from the actual values of the permeability changes (Table 7.5) and noting the locations in the logs where permeability increases were found. These show that the area around the pellet inoculation site (position C) show the highest increases in permeability.

The results of the permeability determinations for the barked log's heartwood region are illustrated in figure 7.8



7.8 Frequency (number of core sections giving an increase in permeability in Scots pine heartwood) of % increases in permeability of barked Scots pine heartwood treated with selected *Trichoderma* isolates.

The heartwood region show increases in permeability in most samples after treatment with the selected *Trichoderma* isolates. When analysed statistically these increases in permeability were significant at 95% confidence limits.

Pine control logs showed generally lower % increases in permeability, this may be due to the protective effect of the bark preventing contaminant organisms from colonising the timber. The effect of the bark also appears to have affected the ability of the organisms to increase the permeability of the outer regions of the timber possibly because of the growth patterns produced.

A representation of the actual permeabilities can be seen in table 7.5

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	559	662	3.65
	N	577	204	10
	N2	184	504	1.7
	C1	1622	1601	16
	C	39	46	22
	C2	1658	1711	2.2
	B1	333	173	1.5
	B	485	292	1.2
	B2	242	473	1.7
T.viride	N1	181	777	1.9
	N	43	62	13
	N2	1820	350	6
	C1	996	1709	24
	C	100	173	2.6
	C2	498	1405	8.8
	B1	1281	88	2.6
	B	28	23	18
	B2	315	785	1.43

Table 7.5 Representative values for radial air permeability from barked Scots pine logs.

The results in table 7.5 shows that logs inoculated with *Trichoderma aureoviride* has the largest increase in permeability around the inoculation site and that in these areas the

permeability values are similar to those seen at each of the sample site locations in the earlier log treatments. The results for *Trichoderma viride* (T35) show that there is a better overall improvement in the permeabilities of the outer sapwood (Depth 1) at sample sites nearest to the central inoculation site (N2, C1, C2 and B1).

The frequency of increased permeability of sapwood samples removed from debarked Scots pine logs treated with selected *Trichoderma* isolates can be seen in figure 7.9.

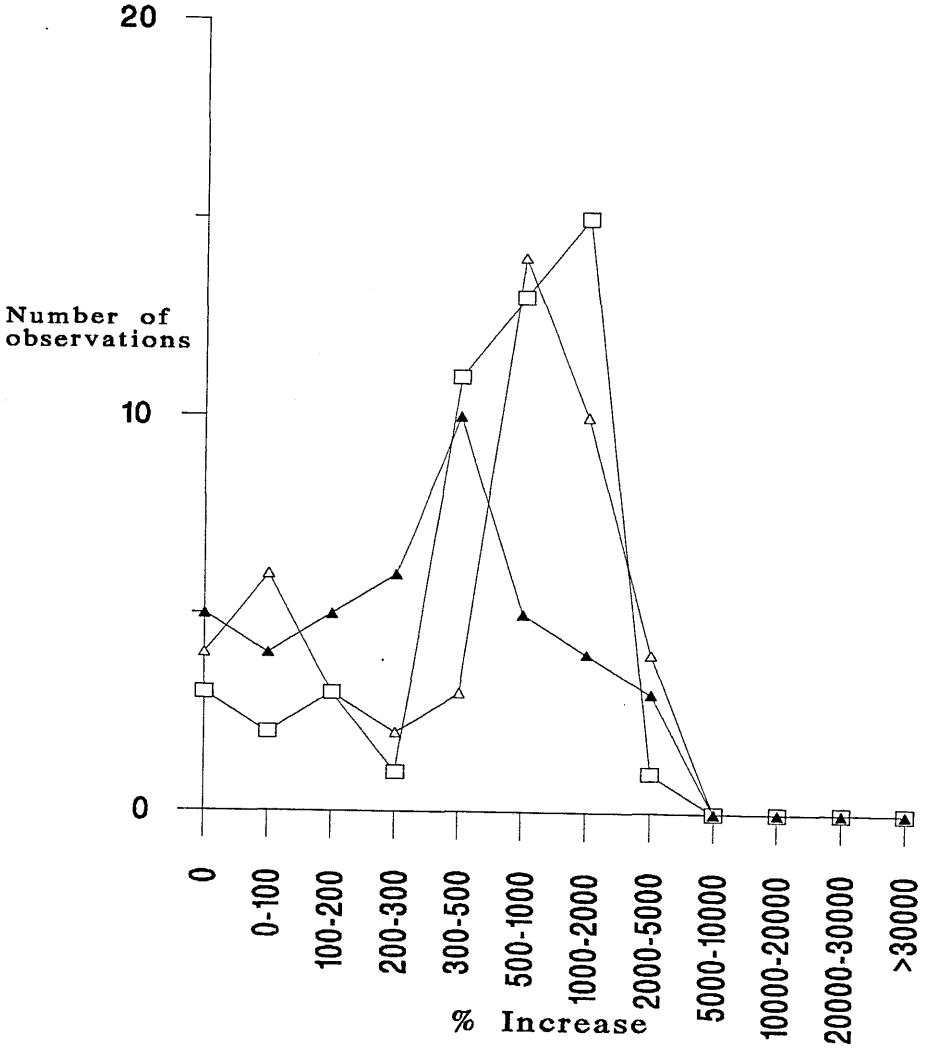


Figure 7.9 Frequency (number of core sections giving an increase in permeability in Scots pine sapwood) of % increases in permeability of debarked Scots pine sapwood treated selected *Trichoderma* isolates.

This figure shows that in the majority of sapwood samples the permeability after incubation with *Trichoderma* is increased. The distribution shows that the majority of sapwood cores showed % increases of between 500-5000%. This figure shows a similar distribution to other treatments with most of the sapwood material showing an increase in permeability. Pine control logs show again generally lower % increases in permeability, this may be due to any other colonising organisms being unable to increase the permeability of the timber to the same extent as the selected isolates. When these results were analysed statistically both *Trichoderma* isolates showed a significant change in permeability when compared to the control material (at 95% confidence limits).

The debarked Scots pine heartwood results are illustrated in figure 7.10

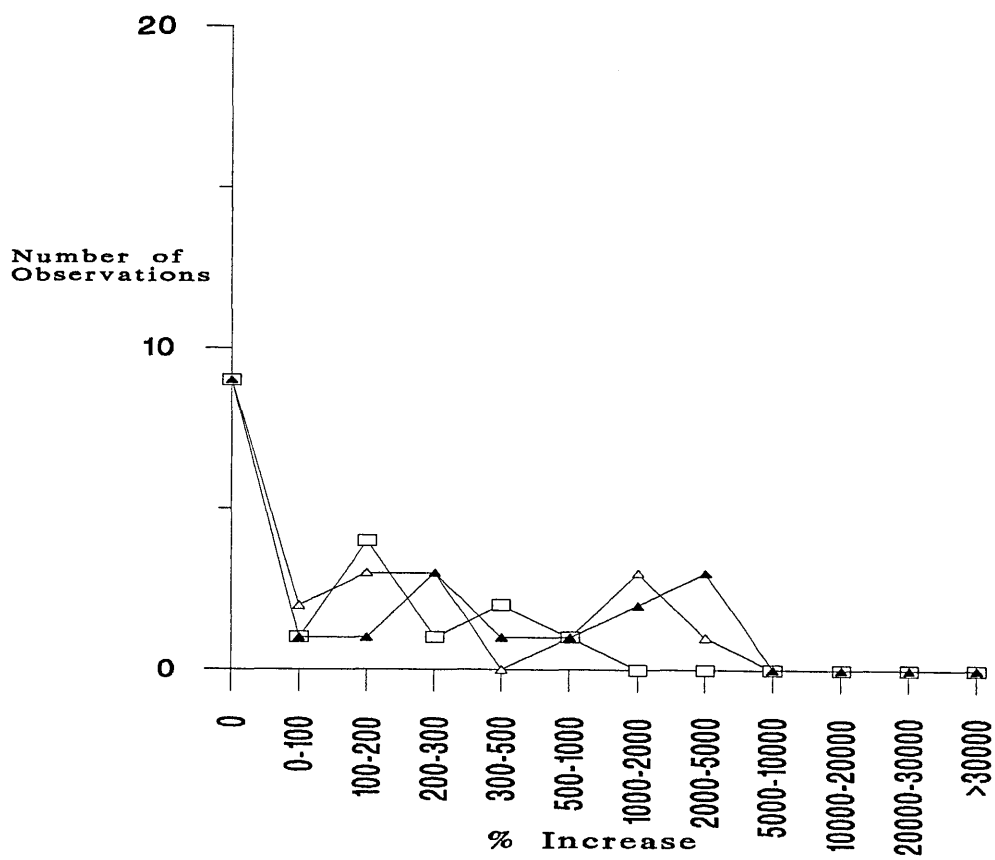


Figure 7.10 Frequency (number of core sections giving an increase in permeability in Scots pine heartwood) of % increases in permeability of debarked Scots pine heartwood treated selected *Trichoderma* isolates.

Heartwood material again shows lower increases in permeability compared to the sapwood and again a large number of samples showed no positive increase. Both isolates appeared to make no significant change to the permeability of the pine heartwood when compared to the control material.

Representative figures showing the actual permeabilities can be seen in Table 7.6.

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	1226	1518	10
	N	58	109	5
	N2	1304	1018	70
	C1	835	687	10
	C	80	49	12
	C2	1000	707	46
	B1	533	322	19
	B	976	322	7
	B2	121	116	12
T.viride	N1	889	579	164
	N	69	60	52
	N2	859	771	5
	C1	1265	745	7
	C	228	111	25
	C2	1026	465	27
	B1	945	689	6
	B	82	48	7
	B2	1390	484	36

Table 7.6 Representative values for radial air permeability from debarked Scots pine logs

The results in table 7.6 show significant increases in most of the sapwood samples irrespective of position on the log and some heartwood samples.

Sitka spruce logs

The frequency of increased permeability in debarked Sitka spruce sapwood from logs treated with 2% fluoride and inoculated with selected *Trichoderma* isolates can be seen in figure 7.11

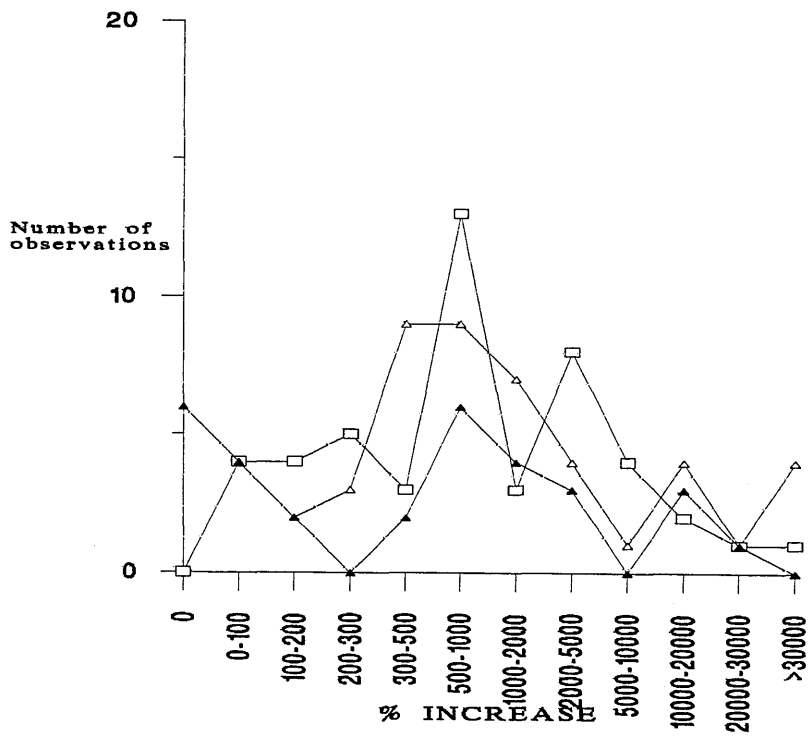


Figure 7.11 Frequency (number of core sections giving an increase in permeability of Sitka spruce sapwood) of % increases in permeability of Sitka spruce logs treated with 2% fluoride and selected *Trichoderma* isolates.

This figure shows that in all sapwood samples the permeability after incubation with the selected *Trichoderma* isolates is increased. Samples from these 8 logs generally show higher % increases compared with some of the pine samples i.e. > 30000%. Although these large % increases in permeability appear much greater than for pine samples it should be noted that the permeabilities are still smaller than the untreated samples in the pine (comparison of tables 7.6 and 7.7). However statistical analysis of the results do not show any significant increases in permeability after treatment with the selected *Trichoderma* isolates compared to the control material.

Results from heartwood samples can be seen in figure 7.12.

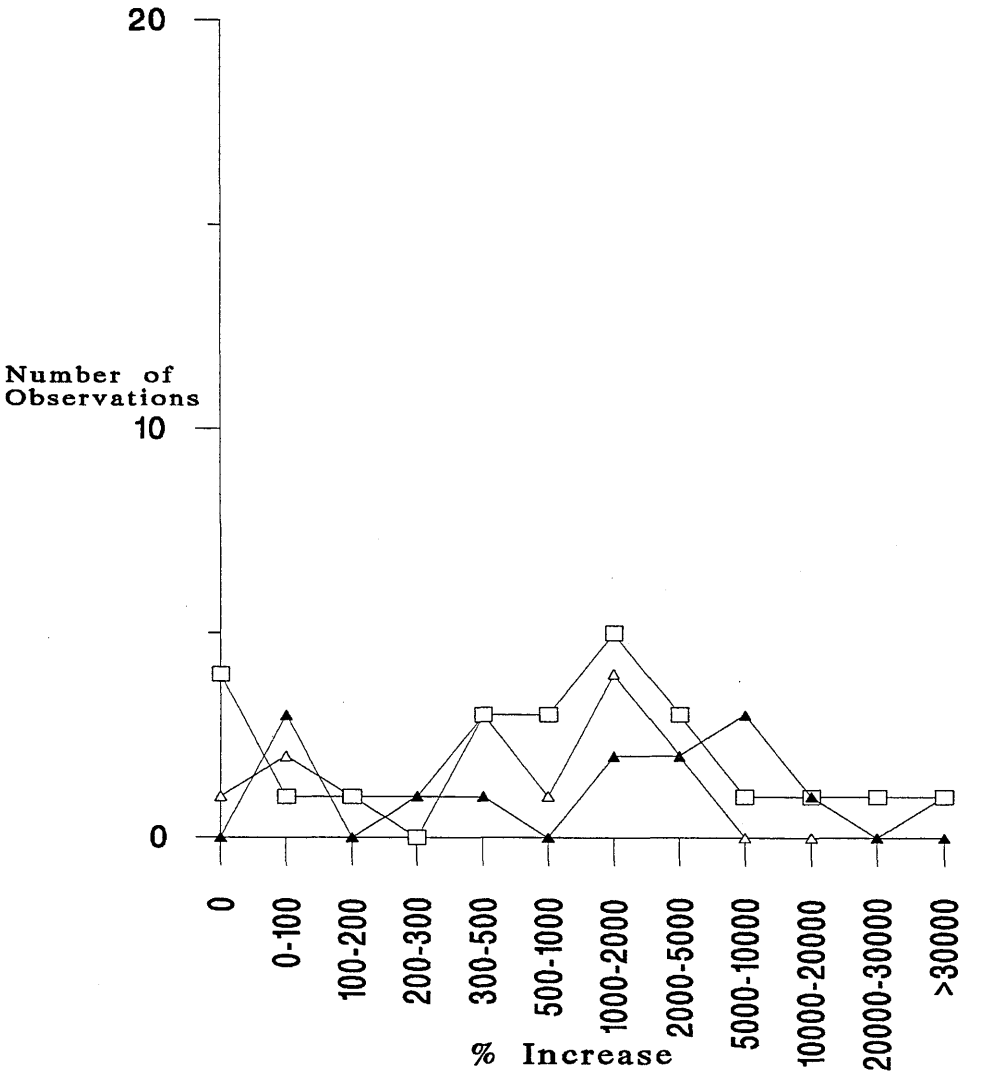


Figure 7.12 Frequency (number of core sections giving an increase in permeability of Sitka spruce heartwood) of % increases in permeability of Sitka spruce logs treated with 2% fluoride and selected *Trichoderma* isolates.

The heartwood material does not show the same distribution as sapwood material with 9 samples giving increases of 5 fold or less while 7 samples gave increases of 50 fold or more.

Control logs show similar increases in permeability compared to those treated with *Trichoderma*. This may be due to the fluoride aiding the colonisation by other *Trichoderma* isolates that increased the permeability to the same extent as seen with the selected organisms.

Representative values for the permeabilities at each depth can be seen in Table 7.7.

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	7.92	13.08	0.47
	N	2.52	0.129	0.03
	N2	13.1	8.18	4.16
	C1	10.63	8.44	0.57
	C	1.523	0.53	0.03
	C2	12.43	10.79	0.35
	B1	9.19	9.64	1.44
	B	0.005	0.99	0.03
	B2	15.45	15.39	0.133
T.viride	N1	11.09	13.38	0.28
	NN2	0.03	5.36	0.21
	C1	7.29	6.39	0.56
	C	10.57	5.35	1.45
	C2	0.07	1.21	0.07
	B1	14.33	8.02	0.31
	B	15.01	13.37	2.026
	B2	1.87	5.321	0.03
		8.04	7.9	0.67

Table 7.7 Representative values for actual permeability measurements from cores removed from Sitka spruce logs treated with 2% fluoride and *Trichoderma* isolates.

The results in table 7.7 show the permeabilities of representative logs. The permeability of this timber is lower than the pine and in most cores remains so even after treatment with the *Trichoderma* isolates. The large % increase in permeability are due to the low starting permeability of some reference cores and the subsequently higher values for the treated timber.

The frequency of increased permeability in Sitka spruce sapwood from debarked logs treated with 8% fluoride and inoculated with selected *Trichoderma* isolates can be seen in figure 7.13.

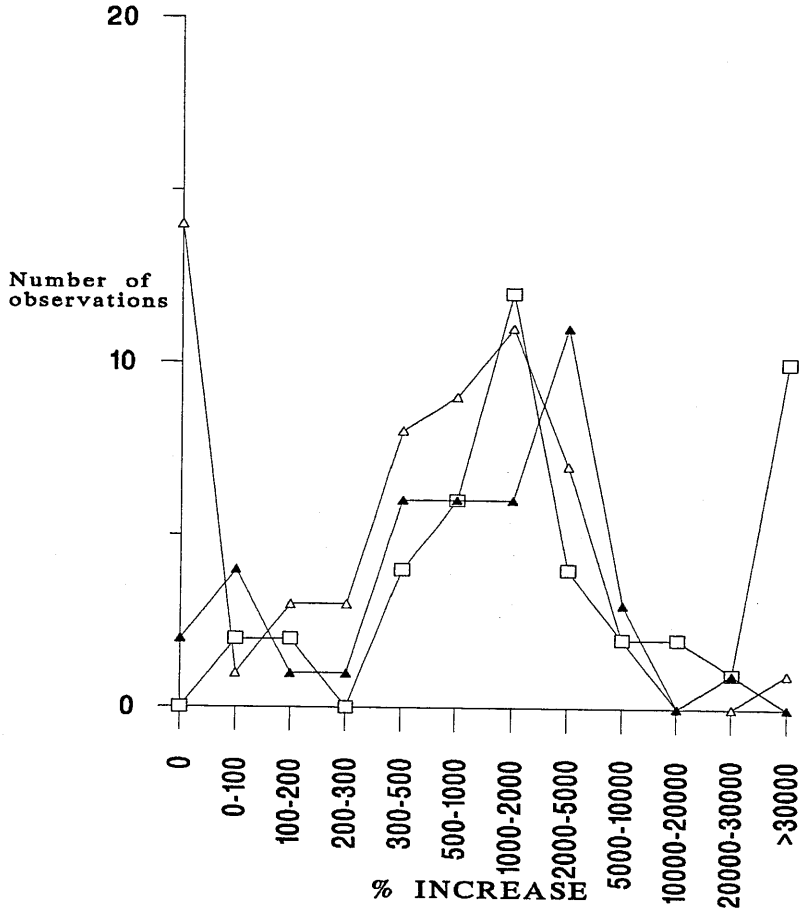


Figure 7.13 Frequency (number of core sections giving an increase in permeability of Sitka spruce sapwood) of % increases in permeability of Sitka spruce logs treated with 8% fluoride and selected *Trichoderma* isolates.

This figure shows that in all but one of the sapwood samples treated with *Trichoderma* the permeability after colonisation was increased. When these samples were compared with the control material there was a significant increase in the sapwood permeability after treatment with *Trichoderma* (at 95% confidence limits).

Results from heartwood samples removed from logs treated with 8% Fluoride can be seen in figure 7.14

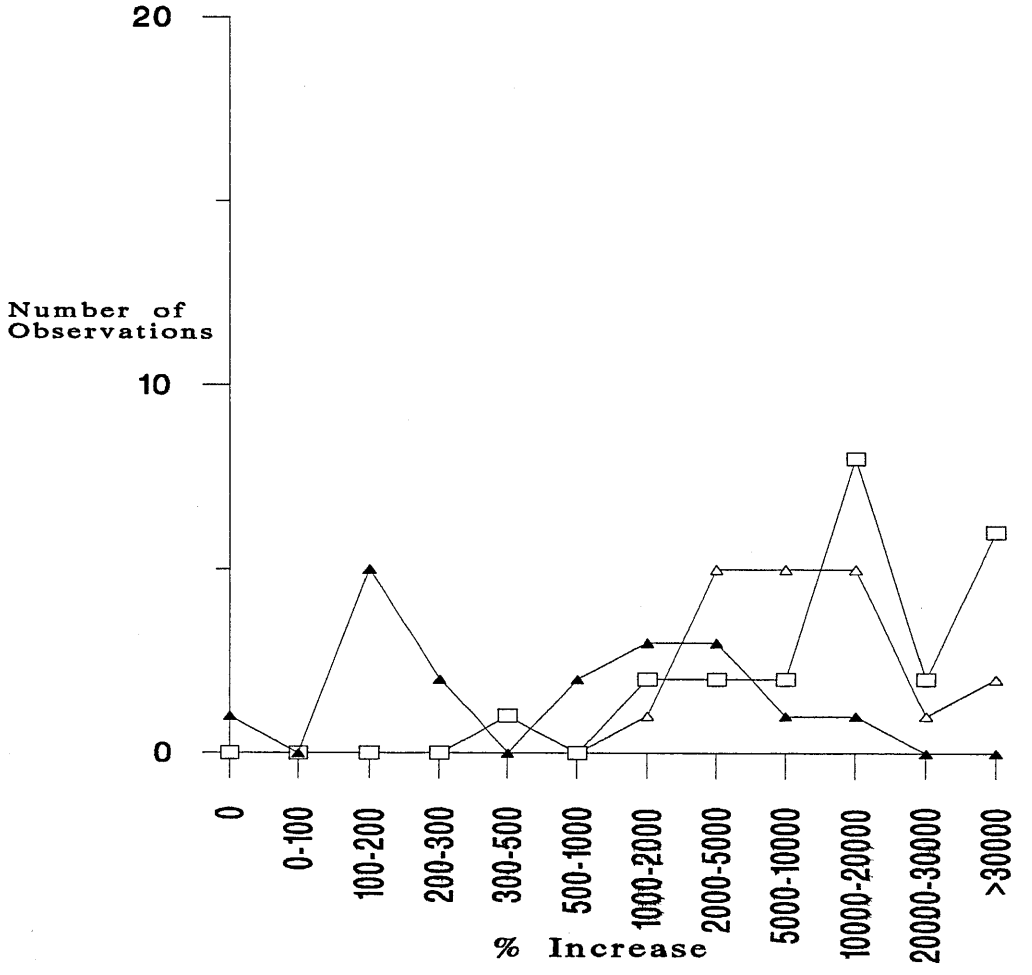


Figure 7.14 Frequency (number of core sections giving an increase in permeability of Sitka spruce heartwood) of % increases in permeability of Sitka spruce logs treated with 8% fluoride and selected *Trichoderma* isolates.

In the heartwood all samples showed an increase in permeability the majority showing very large increases in permeability (5000-20000%) after treatment with *Trichoderma*. Again when these were analysed statistically the permeability was increased significantly (at 95% confidence limits).

The greatest increases in permeability were seen at the sites closest to the inoculation point. This is illustrated in Table 7.8 where representative figures showing the actual permeabilities can be seen.

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	24	18	2.6
	N	0.06	7.3	0.06
	N2	15.96	7.5	8.8
	C1	21.06	21.36	5.69
	C	0.24	7.7	0.08
	C2	19.5	23.5	3.97
	B1	29.34	21	11.06
	B	4.08	5.11	0.1
	B2	12.1	29	6.52
T.viride	N1	16.6	18.31	4.72
	N	0.661	0.72	0.03
	N2	15.19	28.04	3.9
	C1	28.12	47.93	4.13
	C	1.74	0.1	0.04
	C2	108	43.98	34.95
	B1	20.3	26.3	1.93
	B	1.94	0.33	0.03
	B2	27.15	162	2.24

Table 7.8 Representative values for radial air permeability from Sitka spruce logs treated with 8% fluoride.

The results in table 7.8 show that there is large increases in permeability after incubation with the *Trichoderma* isolates, but again the overall values for permeability are much lower than for similarly treated pine samples.

The frequency of increased permeability in sapwood samples of barked Sitka spruce logs treated with selected *Trichoderma* isolates can be seen in figure 7.15.

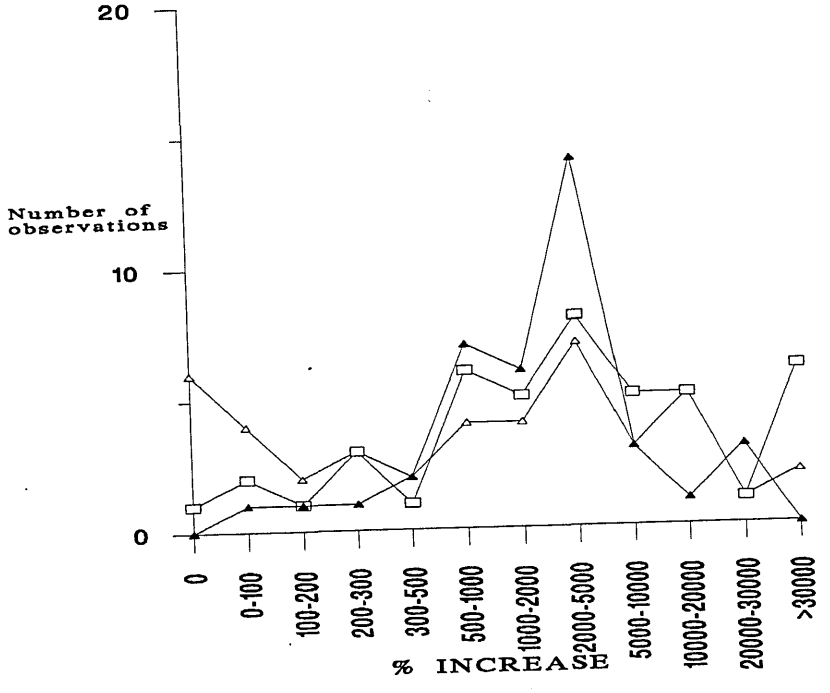


Figure 7.15 Frequency (number of core sections giving an increase in permeability of Sitka spruce sapwood) of % increases in permeability of barked Sitka spruce logs inoculated with selected *Trichoderma* isolates.

The results shown in figure 7.14 show there are increases in the majority of sapwood samples after treatment with the selected *Trichoderma* isolates. When these were compared to the control material it was found that only those samples treated with *Trichoderma viride* SIWT 70 showed a significant increase in permeability (at 95% confidence limits).

The results shown in figure 7.16 show increases in permeability in heartwood material after treatment with selected *Trichoderma* isolates.

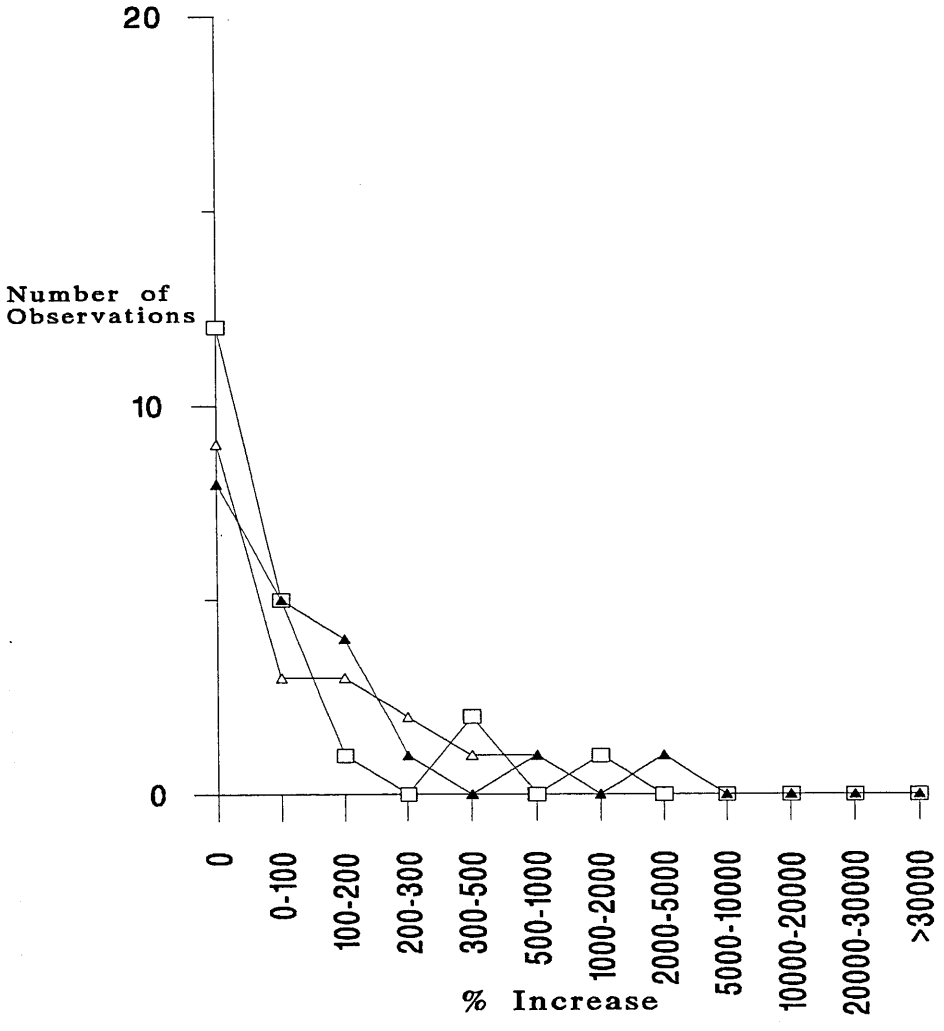


Figure 7.16 Frequency (number of core sections giving an increase in permeability of Sitka spruce heartwood) of % increases in permeability of barked Sitka spruce logs treated with selected *Trichoderma* isolates.

When the results for the cores removed from logs treated with selected *Trichoderma* isolates were compared to the control material there were no significant increases in permeability.

It is again likely that the pattern of colonisation in the logs is responsible for the higher increases in permeability around the inoculation points in the treated timber.
A representation of the actual permeabilities can be seen in table 7.9

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	12.66	5.19	6.33
	N	0.22	0.67	0.37
	N2	22.4	7.78	0.43
	C1	49.78	7.51	4.2
	C	23	1.9	2.34
	C2	11.66	6.24	1.25
	B1	13.39	0.99	1.32
	B	0.53	0.25	7.27
	B2	8.11	5.59	1.92
T.viride	N1	18.34	25.55	4.04
	N	0.59	0.31	0.64
	N2	11.18	63.69	1.65
	C1	19.36	27	4.83
	C	0.544	1.01	8.14
	C2	25.6	144.5	21.59
	B1	10.4	37.04	8.16
	B	0.37	0.34	1.52
	B2	9.96	35.64	4.8

Table 7.9 Representative values for radial air permeability from barked Sitka spruce logs.

This table shows that again after incubation with *Trichoderma* isolates the permeability of the timber has improved in most cases. High % increases in permeability are the result of low starting permeabilities of the core samples as well as the effect of the *Trichoderma* treatment. The permeabilities after *Trichoderma* treatment are still lower than untreated pine material and are broadly similar to those observed with the other Sitka spruce treatments.

The frequency of increased permeability in sapwood samples removed from debarked Sitka spruce logs treated with selected *Trichoderma* isolates can be seen in figure 7.17.

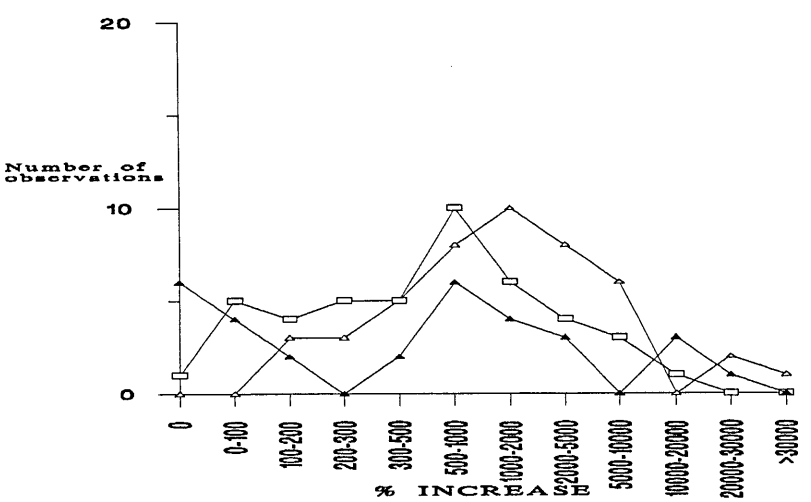


Figure 7.17 Frequency (number of core sections giving an increase in permeability of Sitka spruce sapwood) of % increases in permeability of debarked Sitka spruce logs treated with selected *Trichoderma* isolates.

This figure shows that in the majority of sapwood samples the permeability after incubation with the selected *Trichoderma* isolates has been increased. The figure also shows the wide variation in the extent of increases in permeability between cores. When these were analysed statistically and compared to the control material the increases in permeability after incubation with the *Trichoderma* isolates were found to be significant (95 % confidence limits).

The results showing increases in permeability for heartwood material removed from logs treated with selected *Trichoderma* isolates and can be seen in figure 7.18.

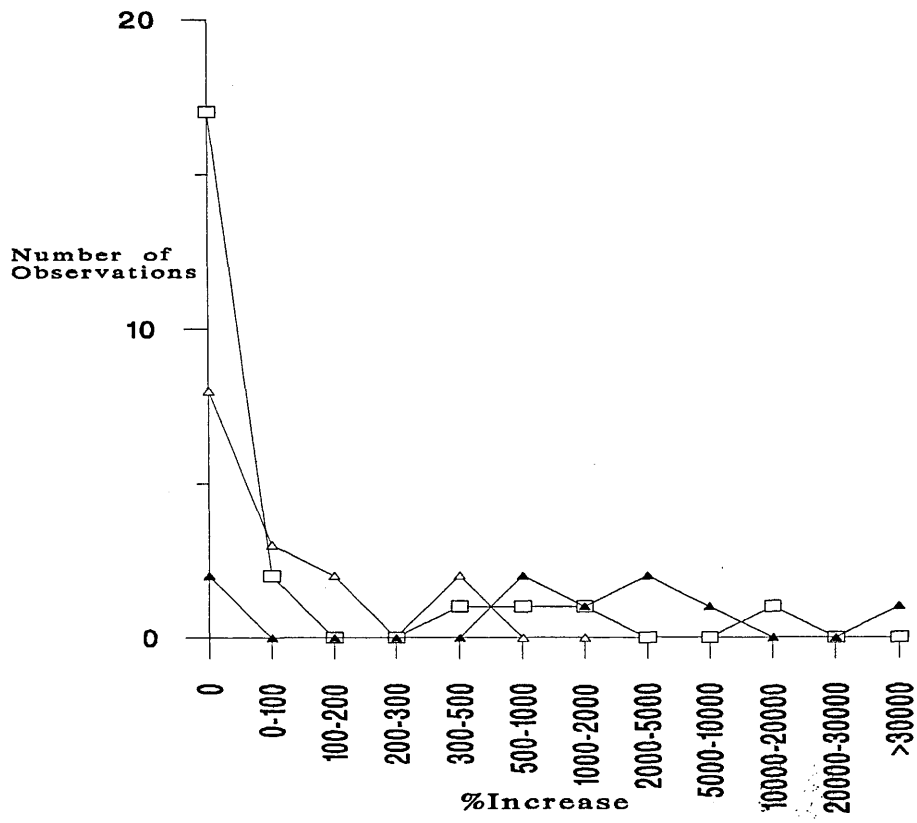


Figure 7.18 Frequency (number of core sections giving an increase in permeability of Sitka spruce heartwood) of % increases in permeability of debarked Sitka spruce logs treated with selected *Trichoderma* isolates.

The heartwood material again shows lower increases in permeability compared to the sapwood and again a large number of samples showed no positive increases. When these results were analysed statistically, the increases in permeability observed for *Trichoderma* isolates were found not to be significantly different from the control material.

Representative figures showing the actual permeabilities can be seen in Table 7.10.

Trichoderma isolate	Core position	Sapwood Depth 1 (Darcys x 10^4)	Sapwood Depth 2 (Darcys x 10^4)	Heartwood (Darcys x 10^4)
T.aureoviride	N1	10.81	16.28	5.25
	N	0.68	1.54	1.84
	N2	8.34	12.62	0.25
	C1	8.61	23.77	2.60
	C	1.02	0.11	2.03
	C2	17.6110.72	6.78	2.85
	B1	0.41	7.66	0.81
	B	13.84	1.24	0.68
	B2		26.79	0.38
T.viride	N1	67.24	10.7	0.82
	N	9.13	10.59	1.79
	N2	69.36	12.8	3.3
	C1	83	80.27	8.35
	C	1.382	6.13	0.74
	C2	78.98	2.48	0.16
	B1	31.88	4.63	1.63
	B	5.52	1.08	0.31
	B2	41.3	5.04	2.64

Table 7.10 Representative values for radial air permeability from debarked Sitka spruce logs after treatment with *Trichoderma* isolates.

The results in the above table show the permeability values for debarked spruce logs. The values are broadly similar to those seen in earlier treatments and are again lower than those seen with Scots pine samples.

The moisture contents of the samples were determined prior to air permeability analysis. These were initially around 130% for pine sapwood dropping to 50% by the end of the experiment. Heartwood material ranged between 25 and 60% at the start of the incubation with little difference observed by the end of the experiment. For spruce sapwood moisture contents were initially around 230% dropping to 130% by the end of the experiment.
A summary of the statistical analysis for the different treatment regimes can be seen in Table 7.11.

	Scots pine		Sitka spruce	
Log Treatment	*Trichoderma aureoviride* SIWT1	*Trichoderma viride* SIWT70	*Trichoderma aureoviride* SIWT1	*Trichoderma viride* SIWT70
Sapwood				
Barked	+	*	-	*
0% Fluoride	*	*	*	*
2% Fluoride	+	-	-	-
8% Fluoride	*	*	*	*
Heartwood				
Barked	*	-	-	-
0% Fluoride	-	-	-	-
2% Fluoride	*	+	-	-
8% Fluoride	*	*	*	*

Table 7.11 Summary of Analysis of variance between *Trichoderma* treated logs and control material. N.B. "*" represents significant increases in permeability at 95% confidence limits, "+" represents significant increases in permeability at 90% confidence limits and "-" represents no significant increases in permeability.

Table 7.11 shows that with most of the treatments to pine sapwood material there was a significant increase in permeability. However there were fewer instances of permeability enhancement observed in the spruce material which may be linked to the lower residual permeability of the timber or the greater variation in the % increases observed after incubation with the *Trichoderma* isolates.

7.4 Discussion

From tables 7.2 a and b it can be observed that the roundwood logs were successfully colonised by both *Trichoderma* isolates, which were able to grow in the high moisture contents found initially in the sapwood of the logs. Moisture content is generally seen as in inhibitor to fungal growth (Nicholas and Siau, 1973), however it would appear that the selected *Trichoderma* isolates were able to overcome this barrier and colonise the timber. The isolation of *Trichoderma* from the timber indicates that at least one hyphal strand of the *Trichoderma* has reached the sampling point and does not give an indication of biomass or necessarily mean that the permeability of the timber will be increased.

Different inoculation methods appear to have given rise to different colonisation patterns. The spore suspension used covered a large surface area of the log and permitted a more even colonisation. Using the pellets gave rise to a different growth pattern extending outwards from the single inoculation and a slower colonisation.

Permeability Enhancement

Scots pine material

Barked Scots pine logs treated with pellets of selected *Trichoderma* isolates showed the highest increases in permeability around the inoculation sites while in other areas permeability increases were not as high (Tables 7.5). With *Trichoderma aureoviride* SIWT1 the increase in permeability in the outer sapwood was restricted to cores removed from the sites immediately adjacent to the inoculation point, even though increases in the permeability of the inner sapwood was recorded at most points. This pattern of increase is likely to be due to the ballooning of growth from the inoculation site where the inner regions are more heavily colonised than the outer regions hence the permeability is increased more in this region (figure 7.19). Barked Scots pine logs inoculated with

Trichoderma viride (SIWT 70) however showed improved permeability throughout the central region of the logs (N_2, C_1, C_2 and B_1) which may indicate better growth of the *Trichoderma* isolate in the outer regions of the logs. As with the *Trichoderma aureoviride* (SIWT 1) treatment the inner sapwood permeability was also increased in most samples.

From these results improvements in permeability appear to be dependent on the biomass of the organism that is growing through particular areas of the wood as areas next to inoculation points show the greatest increases in permeability. Given that the organisms will use the most readily available nutrients before more complex compounds, it would be reasonable to assume that initial growth through timber would be dependent on compounds such as sap sugars but as the organisms grow and these become depleted then they will have to switch to other compounds to continue to grow. Structures that limit the permeability of timber are composed of pectin and cellulose. If the organisms are to destroy these structures then they must produce the enzymes to breakdown the material. Hence as the organisms grow they will increase biomass and breakdown more of the structures that restrict permeability, but only once the readily available simple sugars are exhausted.

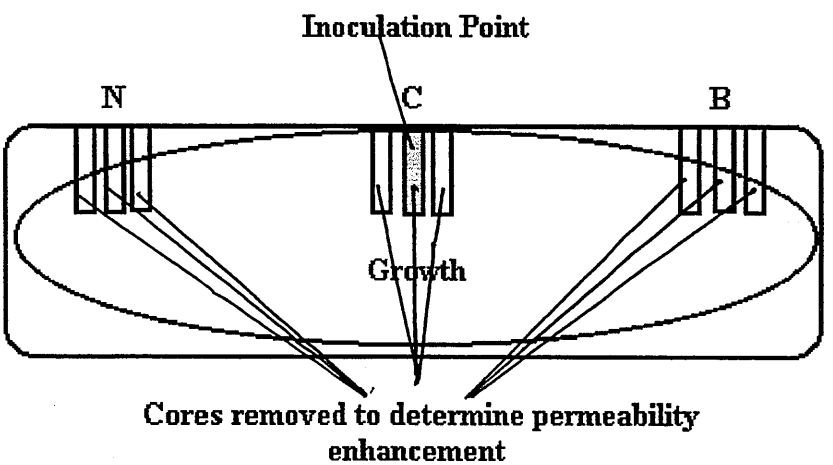


Figure 7.19 Hypothesised growth from pellet inoculation of pine logs

Figure 7.7 shows the distribution of different increases in permeability for the barked pine logs, it appears that with the bark left intact the permeability of the outer regions is altered to a lesser extent than the inner regions. This may be due to the pattern of growth described earlier or may be due to physiological conditions in the outer regions as timber remaining barked should remain active for longer periods than material that is debarked and thereby limit colonisation.

Fluoride has historically been used as a component of chemical wood preservatives (Morrell and Love, 1995). Lindgren and Harvey (1952) also showed that a low fluoride concentration (2%) actively encouraged *Trichoderma* growth. Since *Trichoderma spp.* are

generally seen as pioneer organisms it is likely that they can survive in areas where other organisms are excluded, hence the presence of fluoride would restrict the growth of competing organisms "encouraging" *Trichoderma* to grow and colonise disturbed sites more effectively.

Control pine logs treated with 2% NaF showed some unidentified *Trichoderma* colonisation (Table 7.2a). This growth did not appear to substantially increase the permeability of the timber (compared to those spray inoculated with *Trichoderma aureoviride* SIWT1) (figure 7.3 and 7.4) and it is assumed that these contaminant isolates were not as efficient permeability enhancers. Since 2% Fluoride has allowed surface contamination by foreign *Trichoderma* isolates its use in field experiments may adversely affect the performance of selected isolates through competition, particularly if foreign *Trichoderma* isolates establish themselves as the pioneer organism.

Results from the *Trichoderma* treated logs using 8% fluoride are significantly better than control logs (figures 7.5 and 7.6). This may be due to the high % of fluoride inhibiting natural colonisers in the control logs thus producing a much lower change in permeability than would otherwise be expected. The pine material shows good increases in the sapwood region again particularly around the inoculation points (table 7.4), however the use of pellet inoculum appears to be a less effective for colonisation as the time taken for the organism to grow from the pellet to fully colonise the log will be increased compared to spore inoculation.

Debarked *Trichoderma* treated Scots pine material showed similar increases in permeability to those treated with 2% NaF and debarked control material also showed significantly fewer increases in permeability compared to those treated with *Trichoderma* isolates. Debarking material mirrors the procedure currently used prior to seasoning timber for pole production, the introduction and establishment of organisms that can

improve the permeability of the timber during seasoning should therefore enhance future preservative treatment.

Pine heartwood samples showed significantly lower permeabilities compared to sapwood material. After treatment with the selected *Trichoderma* treatments there was a significant increase in the permeability of some pine heartwood samples. On completion of the incubation with the selected *Trichoderma* isolates the final permeability of the heartwood however remains low and it may be that these changes will not significantly increase preservative treatment into this region.

Sitka spruce material:

Spruce material showed a noticeably different distribution of permeability increases compared to the pine material. There was no difference between the inner and outer sapwood values for any of the treatments with most showing an increase in permeability between 1000 and 2000%. The permeability values observed for the spruce and pine material are broadly similar to those recorded by Siau (1984)
(Tables 7.3-7.10). However the % increases in permeability are generally much greater than those observed for pine samples (Figures 7.3-7.10) and are linked to the low starting permeability. From such a low value any measurable increase will be of a greater magnitude than that recorded for pine.

Barked spruce samples treated with *Trichoderma viride* SIWT 70 (figures 7.15 and 7.16) showed a significant increase in all sapwood material. Material treated with *Trichoderma aureoviride* SIWT 1 showed no significant increases in permeability compared to the control material. Similar patterns of growth appear to have been expressed with this pellet inoculation as seen with the pine material (tables 7.4 and 7.5). This may be associated with the colonisation of the logs by the organisms. All of the logs treated with *Trichoderma* showed the greatest improvements around the inoculation site,

but at other sample sites the changes in permeability were not as marked or always significant. Despite the results for *Trichoderma aureoviride* SIWT 1 not showing a significant increase in permeability throughout the log the organism did produce a marked improvement next to the inoculation site and does appear capable of improving the permeability of spruce material (tables 7.8 and 7.9).

Material treated with 2% NaF (figures (7.11 and 7.12) showed no significant increases in permeability for all *Trichoderma* treated logs compared to control material. Since contaminant isolates were observed in control logs, it is likely that the control material has shown an improved permeability similar to those observed with the selected *Trichoderma* isolates. This would explain why the results of the 2% NaF treated logs were not significant. The selected organisms were still improving the permeability of the timber, but the improvements where masked by the actions of contaminant *Trichoderma* and other organisms on the control material. It is possible that the contaminant organisms were infact either of the selected *Trichoderma* and hence no improvements would be significant.

Spruce logs treated with 8% fluoride showed the largest frequency of improvements in permeability (figures 7.13 and 7.14). This appears to indicate that the application of fluoride improved the % increase in permeability but actual final values of permeability are not significantly higher after this treatment than with any of the other treatments (Table 7.8). Fluoride from the treatment will diffuse into the wet wood (Morrell and Love, 1995) and with spruce having a higher moisture content the fluoride may have been able to diffuse further into the heartwood region stimulating the *Trichoderma* and giving rise to a more even colonisation and subsequently higher frequency of permeability enhancement. This may cause the stimulation of contaminant organisms and although the results of this experiment indicated that there was a significant increase in permeability, if this system were used in the field it may be that other non-effective *Trichoderma* or other mould/ sapstain organisms could colonise the timber because of the added fluoride, potentially giving rise to colonisation problems for selected *Trichoderma* isolates.

Spruce sapwood removed from debarked material inoculated with the selected *Trichoderma* isolates showed significant increases in permeability compared to similar control material. From the colonisation results it is likely that if the controls remain uncontaminated by *Trichoderma* then the comparative permeability will show that the selected *Trichoderma* isolates are capable of improving the permeability of the timber.

For Spruce material it is likely that changes in permeability are more important in the sapwood region than the heartwood as when treated with preservative the sapwood region must first be penetrated before the heartwood is reached. To this extent it appears that increases in permeability by *Trichoderma aureoviride* SIWT1 are more likely to result in a better product after preservation than that treated with *Trichoderma viride* SIWT 70.

Increasing permeability of timber has previously been shown to increase the penetration of preservative and the loadings achieved in the timber. Historically spruce material has been ponded or sprayed with water to encourage bacterial breakdown of the structures that limit the permeability of the timber (Dunleavy and Fogarty, 1971). Work for the Electricity Boards (Anon, 1983) showed that preservative loadings and penetration were significantly improved by ponding. Tesoro *et al* (1966) showed a relationship between improving permeability and creosote treatment. The results of the permeability investigations (Chapter 4) indicated that more permeable material would absorb more liquid. Given that creosote is liquid it is likely that it will follow this relationship and larger amounts would be absorbed into more permeable timber. Further evidence of more permeable timber absorbing larger amounts of preservative was demonstrated by Hainey (1992). Hainey reported large amounts of Copper Chrome and Arsenic (CCA) preservative being absorbed into different timber species, Corsican pine>Scots pine > Norway spruce > Sitka spruce. This is a direct correlation between the respective permeabilities of the timber species (Comstock 1970; Hainey 1992).

The results from pine logs treated with selected strains indicate that huge improvements in the permeability can be made and this may improve the treatability of the logs since the upper sapwood layers should be more easily penetrated by fluids such as creosote. The large increases in spruce permeability were encouraging compared to reference core samples, but the final actual permeability measurements did not approach the same values from untreated pine sapwood samples. Indeed actual values for improved sapwood permeability in Sitka spruce are more similar to those of untreated Scots pine heartwood. With these increases in spruce permeability it is likely that preservative will penetrate further into the wood but whether this is sufficient to adequately treat the timber will only be answered after creosote treatment when this *Trichoderma* treatment is used on pole material under field conditions.

Problems with permeability enhancement by the *Trichoderma* appears to be linked to the organisms ability to grow throughout the timber. The moisture contents of the timber were not limiting to colonisation by the *Trichoderma* isolates. Initial moisture contents for the pine were around 120% for the pine sapwood and 220% for the Spruce sapwood. These moistures dropped over the incubation period to around 80% for pine sapwood and 110% for spruce sapwood. These moistures did not limit the growth of the *Trichoderma* isolates as the organism was re-isolated from all regions of the logs during the incubation. However in growing through the timber the organisms may be exposed to different extraneous compounds that can inhibit the production or action of enzymes necessary (see chapter 6) to breakdown wood constituents and although the organism is isolated from the wood samples the effect that it is having on the permeability may be reduced and cannot be guaranteed.

Overall the different treatment regimes all produced similar permeability increases where the *Trichoderma* had grown through the timber and the end values for permeability were similar after each of the treatments. Pine logs treated with *Trichoderma aureoviride* (SIWT1) appeared to show the largest number of significant improvements in both the

sapwood and heartwood material. However significant improvements were observed in some of the pine logs treated with *Trichoderma viride* (SIWT 70). Comparing the performance of the two isolates on the spruce material, there appears to be a reversal in ability between the isolates where *Trichoderma viride* (SIWT 70) shows a larger number of significant increases in permeability when compared with control material. Although *Trichoderma aureoviride* (SIWT 1) did improve the permeability of spruce material on a few occasions the increases were not as frequent as with the former isolate. The actual values of the permeability after *Trichoderma* treatment however are similar between the two isolates.

The different inoculation methods and treatments affected the growth and colonisation by the two isolates. Spore inoculation covered a larger surface area of the log and where the *Trichoderma* established it was more effective than growth from the site of pellet inoculation. Since a larger surface area was covered by the spore suspension it is expected that this would result in a more even colonisation of the logs. The use of fluorides to encourage the growth of the *Trichoderma* may also encourage other isolates which will compete with the selected *Trichoderma* and hence may not always give rise to the required increases in permeability.

The results of this trial were used to select the most appropriate delivery method and treatment for investigation of the effect of *Trichoderma* isolates on the permeability of pole material in the field. Given that most pole material is debarked prior to seasoning and that the colonisation of logs left barked was not as complete as debarked material, it was decided that the field trial would be carried out on debarked material.

The methods of inoculation showed that a spore suspension was more effective in achieving full colonisation of the whole log compared to pellet innoculum. Pretreatment of the logs with fluoride gave rise to possible complications with the growth of

contaminant mould or sapstain organisms and subsequent improvements in permeability were not significantly better than those achieved without an application of fluoride.

Hence on the basis of these results it was decided to use the simple debarking and spore inoculation method on the pole material to be used in the field trial. Since both isolates showed similar increases in permeability the two *Trichoderma* isolates were included for the field trial.

Chapter 8. Field Trial of Pole Sections.

8.1 Introduction:

Results in previous chapters on pine and spruce have shown that selected *Trichoderma* isolates can improve the permeability of treated timber. Earlier experiments with small wood samples had shown that the permeability of these samples can be increased after incubation with *Trichoderma* isolates, however these experiments were conducted under laboratory not field conditions. Experimental conditions in the laboratory can be closely controlled and can ensure a constant growth environment for organisms. In the field the material is exposed to constantly altering conditions and these fluctuations will affect the colonisation and growth of the organisms throughout the timber. This series of experiments was designed to investigate the effect of the selected *Trichoderma* isolates on timber under field conditions.

The aim of these experiments was to investigate the effect of two selected *Trichoderma* isolates on permeability, creosote penetration and retention into roundwood pole sections prepared from Scots pine and Sitka spruce poles.

8.2 Methods:

8.2.1. Treatment & Colonisation of Pole Sections

Sections of Scots pine and Sitka spruce (2m long) which met with the standard diameter dimensions for medium pole material (20-30 cm) (ESI 43-88, 1987) were purchased from the Forestry Commission. These were then debarked at James Jones and Sons Ltd, Kirriemuir, Scotland, prior to treatment with the selected *Trichoderma* isolates. Unfortunately due to problems with pole transportation the debarked pole sections were left for 3-4 weeks. This delay would have resulted in the timber starting to dry out prior to *Trichoderma* inoculation and may have hindered the colonisation of the timber. The treatments carried out were as follows:

1) Thirteen pole sections of each timber species were brushed with a spore suspension of *Trichoderma aureoviride* SIWT1 (8×10^7 spores/ ml) , and then covered with a tarpaulin.

2) Thirteen pole sections of each timber species were painted with a spore suspension of *Trichoderma aureoviride* SIWT1 (8×10^7 spores/ ml), and then left uncovered to season.

3) Thirteen pole sections of each timber species were painted with a spore suspension of *Trichoderma viride* SIWT 70 (9.2×10^7 spores/ ml), and then covered with a tarpaulin.

4) Thirteen pole sections of each timber species were painted with a spore suspension of *Trichoderma viride* SIWT 70 (9.2×10^7 spores/ ml), and then left uncovered to season

5) Thirteen pole sections of each timber species were dipped in an anti sapstain "Antiblu" containing 10.5% (w/w or 135g / l) Methylene Bis Thiocyanate and 12.5% 2-(Thiocyanomethylthio)benzothiazole (Hicksons Chemical Ltd) and left uncovered to season

6) Thirteen pole sections of each timber species were also left untreated to season.

The poles treated with the different *Trichoderma spp.* were stacked at different ends of the field site at Tealing (by Dundee, Scotland) approximately 200 meters apart to prevent possible cross contamination. Treatments 5 and 6 were stacked at Kirriemuir to prevent any chance of cross contamination of controls with the *Trichoderma* isolates used at Tealing.

From each treatment, three pole sections of each timber species were randomly selected for sampling purposes. These pole sections were cored prior to inoculation for future air permeability determinations. These cores were then frozen at -20°C until air permeabilities were evaluated. After inoculation cores were removed to evaluate *Trichoderma* colonisation, moisture content and dehydrogenase activity in the sample

pole sections. This sampling was repeated periodically to determine the extent of the incubation period required prior to preservative treatment. These results indicated when the timber had been fully colonised by the *Trichoderma*, after which time extra incubation time may not be advantageous and therefore seasoning could begin. It should be noted that those poles used for sampling were not included in subsequent preservative treatment as the sampling core holes would affect preservative penetration.

Due to the extremely hot weather conditions experienced during the initial setting up period, and the fact that the pole sections had become partially dried before *Trichoderma* inoculation, the uncovered material was sprayed with water to raise the surface moisture content of the pole sections and allow the *Trichoderma* to establish and grow throughout the pole sections. One week after inoculation four cores were removed from each pole section to establish the depth of *Trichoderma* colonisation of the timber. Each of the sample cores was section into four pieces; two sapwood and two heartwood. Each section was placed onto 3% MEA and incubated at 25^{o}C and any subsequent growth was noted. At this time it was apparent that colonisation was poor in uncovered samples (see table 8.1A). After a further 3 weeks chips were aseptically removed from just below the surface of the uncovered pole sections and plated out on 3% Malt Extract Agar (MEA) to establish the extent of surface colonisation in the pole sections. The subsequent growth showed that less than 33% of the pole sections had been colonised by the *Trichoderma*. The uncovered pole sections were then sprayed down with water for 2 hrs and covered with tarpaulins to raise the moisture content and surrounding humidity of the wood. This was repeated 4 days later before these pole sections were reinoculated (6.2 x 10^7 spores / ml, *Trichoderma aureoviride*. 6.2 x 10^7 spores / ml, *Trichoderma viride*) with the appropriate *Trichoderma* isolate. The tarpaulins were left in place until colonisation of the pole sections was established. The groups of pole sections which were initially covered (Treatments 1 & 3) showed good colonisation by *Trichoderma* (Table 8.1B) and therefore did not require to be reinoculated or rewetted. The initially uncovered pole sections

(treatments 2 & 4) were resampled by core inoculation after a further 2 weeks to determine the extent of the *Trichoderma* colonisation.

The three sample poles were periodically sampled for moisture content and dehydrogenase activity and after 26 weeks all of the pole sections were transported to the preservative treatment company James Jones and Sons Ltd, Leven, where they were stacked in an open shed and left to season and dry down to a suitable moisture content. Once the moisture contents were below fibre saturation point i.e. %MC < 30% the pole sections were considered ready for preservative treatment. The cut ends of the pole sections that were to be treated with preservative were sealed with Silco-set 101 (supplied by Ambersil UK Ltd) (as described in ESI 43-88, 1987) to prevent endgrain penetration during preservative treatment.

After 46 weeks 5 of the 10 end sealed pole sections in each treatment category were commercially treated using the standard Reuping process (Desch, 1981) and analysed for preservative penetration and loading in the treated areas. After preservative treatment sections were crosscut by chainsaw into 4 equal lengths to measure the depth of creosote penetration and subsequent preservative loadings.

Pole sections used to sample moisture content and dehydrogenase activity were also used to detect changes in the air permeability on completion of the seasoning. These sample pole sections were used to indicate what was occurring within the other logs in terms of moisture content, dehydrogenase activity and air permeability, since sampling of the other pole sections would have influenced the subsequent patterns of creosote uptake.

8.2.2 Dehdrogenase Activity.

This activity was assessed using a modified dehydrogenase assay (Mowe, 1983). Three cores removed from equidistant points along the length of each pole section were divided into their sapwood and heartwood sections and finely chopped to give pooled 1 gram samples of each wood type from each pole section. These samples were placed in sterile

tubes into which 15 mg of calcium carbonate and 2 ml of triphenyl-tetrazolium chloride (TTC) were added and the samples were mixed vigorously. The tubes were then incubated in the dark at 30°C for 24 hrs. After incubation 5 ml of methanol was added and the samples were again mixed vigorously before a further 3 ml of methanol were added. The samples were then centrifuged at 3000 g for 5 minutes. After spinning the absorbance of the supernatant liquid was measured at 485nm and compared to standard solutions containing known amounts of triphenylformazan (TTF). From this absorbency and the dry weight of the wood samples it was possible to determine the microbial activity in the wood samples in terms of product (TTF) formed (μmol/g/hr).

8.2.3 Moisture Content.

Four further cores were removed from the pole sections two at equally spaced points from the topside and two from the underside of the pole section. These cores were sectioned into 1.5 cm lengths and the moisture content of each portion was calculated by first weighing the section and then drying it overnight at 103°C before reweighing the core. Moisture content of cores only containing sapwood or heartwood were evaluated as the portion containing the sapwood/ heartwood interface would give variable moisture contents depending on the amount of each wood type that was present. The % dry weight was calculated as follows:

$$\frac{\text{Wet Weight - Dry Weight}}{\text{Dry Weight}} \times 100$$

8.2.4 Preservative Determinations

The depth of penetration of creosote was measured in Sitka spruce and Scots pine poles by measuring the depth of creosote ingress at each of the exposed cut faces after sectioning. The depth of penetration was measured at 4 compass points (North, South, East and West)

on each of the exposed faces (Since 5 pole sections were cut into 4 pieces this would give 100 measurements per treatment).

Preservative loadings were determined using the method described in American Wood Preservers Association Standard A6-89 (Anon, 1989) (a method for the determination of oil type preservatives and water in wood).

The spruce samples were extracted after using two different sampling methods in order to establish creosote concentration in the entire sapwood and treated zone respectively.

1) Twenty cores were removed with Matsuun augers from each pole section (5 per cross cut section). Cores were cut to a depth of 45mm and pooled for each log, these cores were then placed into a pre-dried and weighed cellulose crucible and extracted as described below for 2 hrs. Pole sections were sampled to a depth of 45mm as this was the mean depth of sapwood established by staining the freshly cross cut ends of a representative sample (4 poles) of the Sitka spruce material with Bromo creosol green (Kutscha and Sachs, 1962).

2) Fifteen cores were removed with the plug cutter from each spruce pole to the full limits of the preservative treated area. These cores were pooled for each log and extracted using the following method:

The creosote retentions in pine material were determined in terms of creosote retention at different depths within the pole sections and as total treatment of the pole sections. This was done as follows:

Twenty cores were removed with Matsuun augers from each pole section (5 per cross cut section). These cores were cut into 10mm lengths along the core which represented 10mm depths into the pole sections. The cores were pooled for each depth and then placed into a pre-dried and weighed cellulose crucible and extracted as described below for 2 hrs.

All samples were extracted with toluene in Soxhlet apparatus. Water was removed and measured by means of a side arm trap (Dean and Stark apparatus).

Samples were prepared by drying and weighing cellulose crucibles, wood samples were then added to these crucibles and reweighed.

Toluene (200 ml) was placed in a round bottom flask and the Soxhlet apparatus assembled, the Dean and Stark side arm was then connected on top of the Soxhlet flask and a condenser fitted above the side arm tube. The soxhlet and Dean and Stark were wrapped in cotton wool to ensure efficient heating of the solvent and thereby allowing the solvent to be driven completely through the apparatus to the condenser and preventing condensation of the solvent before it reached the side arm and ensuring that the water was trapped in the calibrated tube. The apparatus was switched on and allowed to run for 30 minutes prior to samples being placed in the soxhlet. This allowed any residual water to be driven through the apparatus and trapped in the side arm. Samples were then introduced and extracted for 2 hours. Once the extraction was complete the amount of water in the side arm tube was measured and the samples were removed from the soxhlet. Excess toluene was drained off the samples and the crucibles were then stored in a fume cupboard to allow the excess toluene to evaporate, samples were then dried at 105^{o}C overnight before being re weighed. The amount of creosote extracted from the wood was then calculated using the following equation:

Weight of creosote in sample = (Wood sample weight prior to extraction) - (sample weight after extraction)- (weight of water in sample) (Kg).

Knowing the length of samples used and the diameter of the cores the above weight can be converted to Kg/m^3 by :

$$\frac{\text{Weight of creosote (Kg)}}{\text{volume of cores (m}^3\text{)}}$$

The results of these determinations can be seen in figures 8.4-8.8

8.2.5 Permeability Determinations

Permeability changes were determined by removing sample cores from pole sections prior to the inoculation with *Trichoderma* isolates. Cores were removed with a plug cutter as in previous experiments and frozen on return to the university. Holes left in sample sites by the removal of the cores were plugged with Scots pine dowels. On completion of seasoning the sections were again sampled by removing cores from sites adjacent to the original sample points (see chapter 5). The permeability of these cores were measured using the air permeability method described in chapter 5, and compared with permeability of reference cores.

8.2.6 *Trichoderma* Survival After Preservative Treatment.

After the poles sections were treated with creosote two cores were removed from the outside of the pole section to the pith in each of the treated logs. The cores were then sectioned into those areas treated with preservative and those regions showing no visible preservative penetration. Core sections were then plated out on 3% Malt Extract Agar and incubated for 14 days at 25^{o}C to determine if any organisms particularly *Trichoderma* had survived the preservative treatment.

The results of the plates were noted in terms of growth of any organisms from the core sections and whether any sections contained *Trichoderma.*

8.3 Results

8.3.1 Colonisation of pole sections

The following tables show the results of plating out wood core samples removed from the pole material after *Trichoderma* inoculation. The colonisation of cores in the uncovered pole material 1 week after the original inoculation can be seen in table 8.1.A.

		Sapwood		Heartwood	
Pole section		Depth 1	Depth 2	Depth 3	Depth 4
Uncovered Scots pine T35	Pole section 1	2T	1T	-	-
	Pole section 2	1B	-	-	-
	Pole section 3	1T	2T	1T	1T
Uncovered Scots pine T1	Pole section 1	1T	2T	-	1F
	Pole section 2	1F	1F	-	-
	Pole section 3	2T	1F	1T	1T
Uncovered Sitka spruce T35	Pole section 1	1B+1T	-	-	-
	Pole section 2	-	1T	1T	-
	Pole section 3	-	-	-	-
Uncovered Sitka spruce T1	Pole section 1	2T	1T	1T	1T
	Pole section 2	1T	2B	1T	1T
	Pole section 3	1T	1T	1T	1T

Table 8.1.A. Colonisation of uncovered pole material 1 week after *Trichoderma* inoculation N.B. T indicates *Trichoderma* colonisation, B indicates bacterial colonisation, F indicates other fungal growth. 1-4 represents the number of cores from which the re-isolations occurred. Core depths varied between pole sections, sapwood and heartwood regions were identified and cut into approximately equal lengths. Depth 1 relates to sapwood material that was removed from the outermost region of the timber. Depth 2 relates to the sapwood material in the innermost region of the timber. Depth 3 relates to the heartwood material in the outermost region of the timber. Depth 4 relates to the heartwood material removed from innermost region of the timber.

The colonisation of the covered pole material 1 week after inoculation can be seen in Table 8.1.B.

	Sapwood		Heartwood	
Pole section	Depth 1	Depth 2	Depth 3	Depth 4
Covered Scots pine T35 Pole section 1	3T	2T	2T	2T
Pole section 2	2T	2T	1F	-
Pole section 3	3T	2T	1T	-
Covered Scots pine T1 Pole section 1	3T	2T	2T	1T
Pole section 2	4T	2T	1B	1B
Pole section 3	3T	3T	2T	1T
Covered Sitka spruce T35 Pole section 1	3T	1B	2T	1T
Pole section 2	2T	3T	2T	2T
Pole section 3	2T	3T	2T	1T
Covered Sitka spruce T1 Pole section 1	2T	2T	3T	2T
Pole section 2	2T	3T	2T	2T
Pole section 3	3T	2T	3T	1T

Table 8.1.B. Colonisation of covered pole material 1 week after *Trichoderma* inoculation. N.B.. T indicates *Trichoderma* colonisation, B indicates bacterial colonisation, F indicates other fungal growth. 1-4 represents the number of cores from which the re-isolations occurred. Depth 1 relates to sapwood material that was removed from the outermost region of the timber. Depth 2 relates to the sapwood material in the innermost region of the timber. Depth 3 relates to the heartwood material in the outermost region of the timber. Depth 4 relates to the heartwood material removed from innermost region of the timber.

Tables 8.1A & B clearly show that *Trichoderma* has failed to colonise both spruce and pine pole sections which were left uncovered. It can also be seen from table 8.1B that no

such problem was experienced with the material that was covered from the start of the experiment.

After the uncovered material was covered and soaked with water the colonisation by *Trichoderma* isolates was substantially improved as can be seen in table 8.2

	Sapwood		Heartwood	
Pole section	Depth 1	Depth 2	Depth 3	Depth 4
Uncovered Scots pine T35 Pole section 1	4T	4T	3T	3T
Pole section 2	4T	3T	4T	3T
Pole section 3	4T	4T	4T	2T
Uncovered Scots pine T1 Pole section 1	4T	4T	2T	3T
Pole section 2	3T	4T	3F	2T
Pole section 3	3T	3T	3T	3T
Uncovered Sitka spruce T35 Pole section 1	4T	2F+2T	3T	2T
Pole section 2	3T	3T	3T	2T
Pole section 3	3T	3T	2T	3T
Uncovered Sitka spruce T1 Pole section 1	3T	4T	1F+3T	3T
Pole section 2	3T	3T	2T	2T
Pole section 3	3T	3T	3T	3T

Table 8.2. Colonisation of uncovered pole sections after *Trichoderma* reinoculation
N.B. T indicates *Trichoderma* colonisation, B indicates bacteria colonisation, F indicates other fungal growth. 1-4 represents the number of cores out of 4 from which positive isolations were made.

8.3.2 Dehydrogenase

Pine Samples

The dehydrogenase activity of pine sapwood and pine heartwood samples for all treatments and controls over the 36 week incubation periods are shown in figures 8.1 and 8.2. respectively.

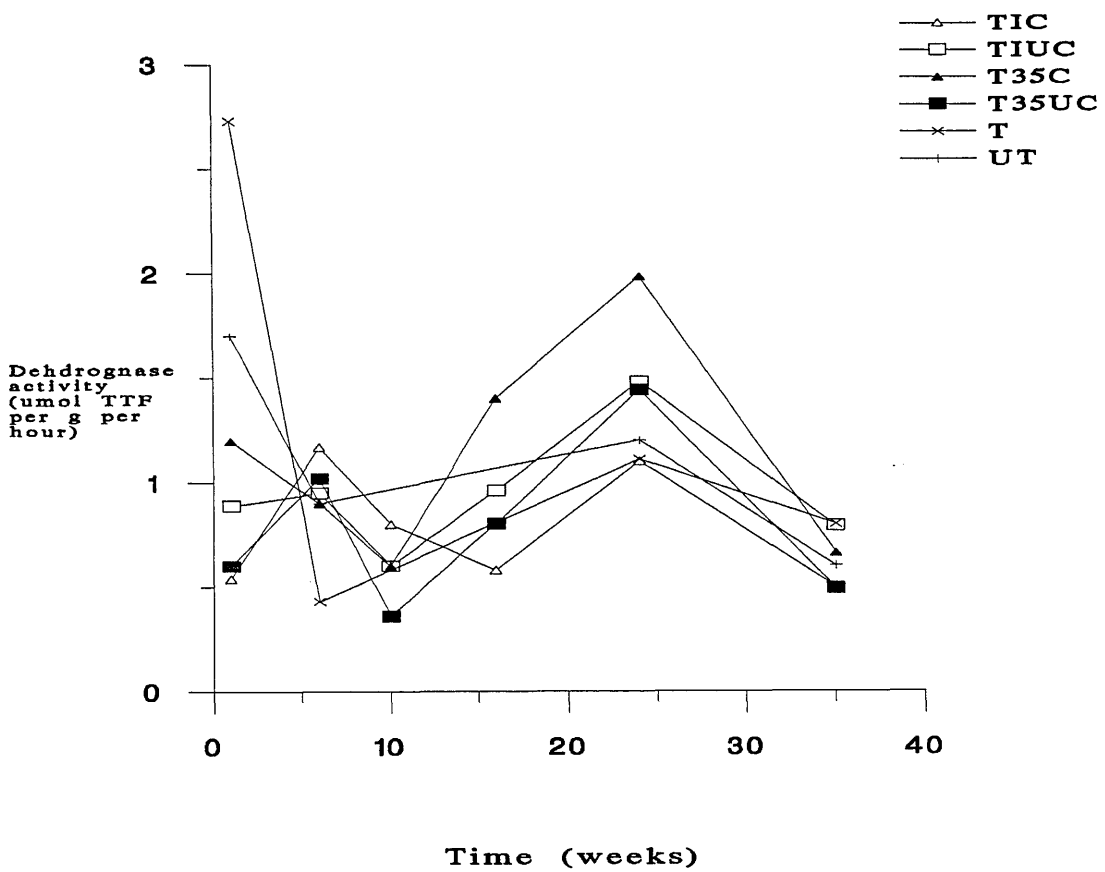


Figure 8.1 Dehydrogenase activity from Scots pine sapwood samples. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB T1C represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1. TIUC represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1. T35C represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70. T35UC represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70. T represents control pole sections that were treated with anti-blu prior to seasoning. UT represents control pole sections that were left untreated prior and during seasoning

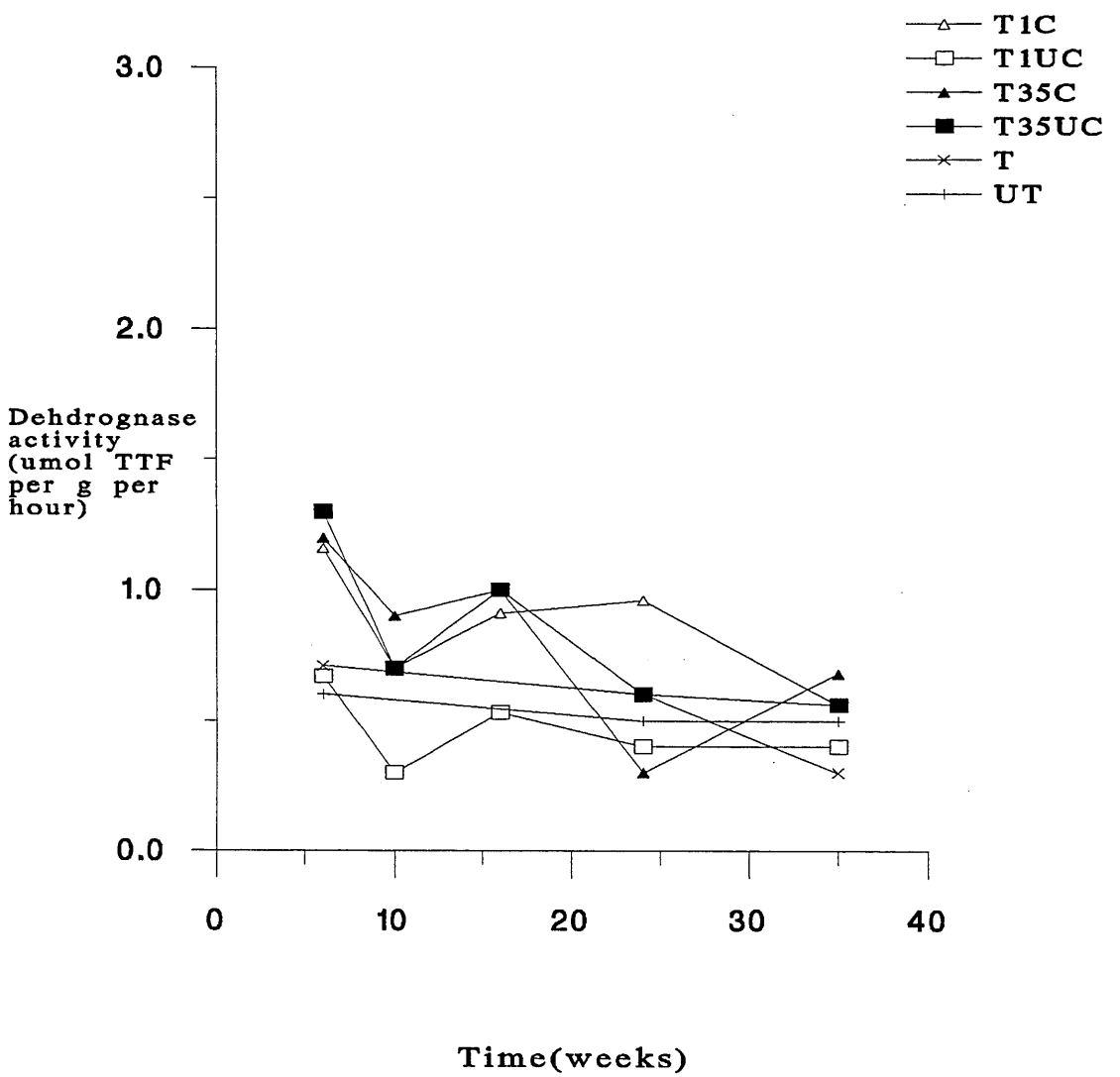


Figure 8.2. Dehydrogenase activity Scots pine heartwood samples. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. T1C represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1. TIUC represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1. T35C represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70. T35UC represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70. T represents control pole sections that were treated with anti-blu prior to seasoning. UT represents control pole sections that were left untreated prior and during seasoning

It is clear that for the uncovered samples the dehydrogenase activity falls from the time of inoculation until the pole material was re-wetted and covered (4 weeks after inoculation) after which the dehydrogenase activity increased. After the covers were finally removed from these poles at 25 weeks the dehydrogenase activity then fell to its final value after 35 weeks.

For all covered pine material there is, after an initial burst of dehydrogenase activity a gradual decline until the 10 week sampling period at which time the covers were removed (figure 8.1). The dehydrogenase activity then increased before declining again by the 35 week sampling interval.

Dehydrogenase activity is generally lower in the heartwood material compared with sapwood. For those pole sections treated with "Anti-blu" the dehydrogenase activity falls dramatically after such treatment at 2 weeks, thereafter showing a slight increase. For the untreated samples dehydrogenase activity decreases progressively throughout the incubation period.

The results of the dehydrogenase assay from Sitka Spruce sapwood and Heartwood can be seen in figures 8.3 and 8.4

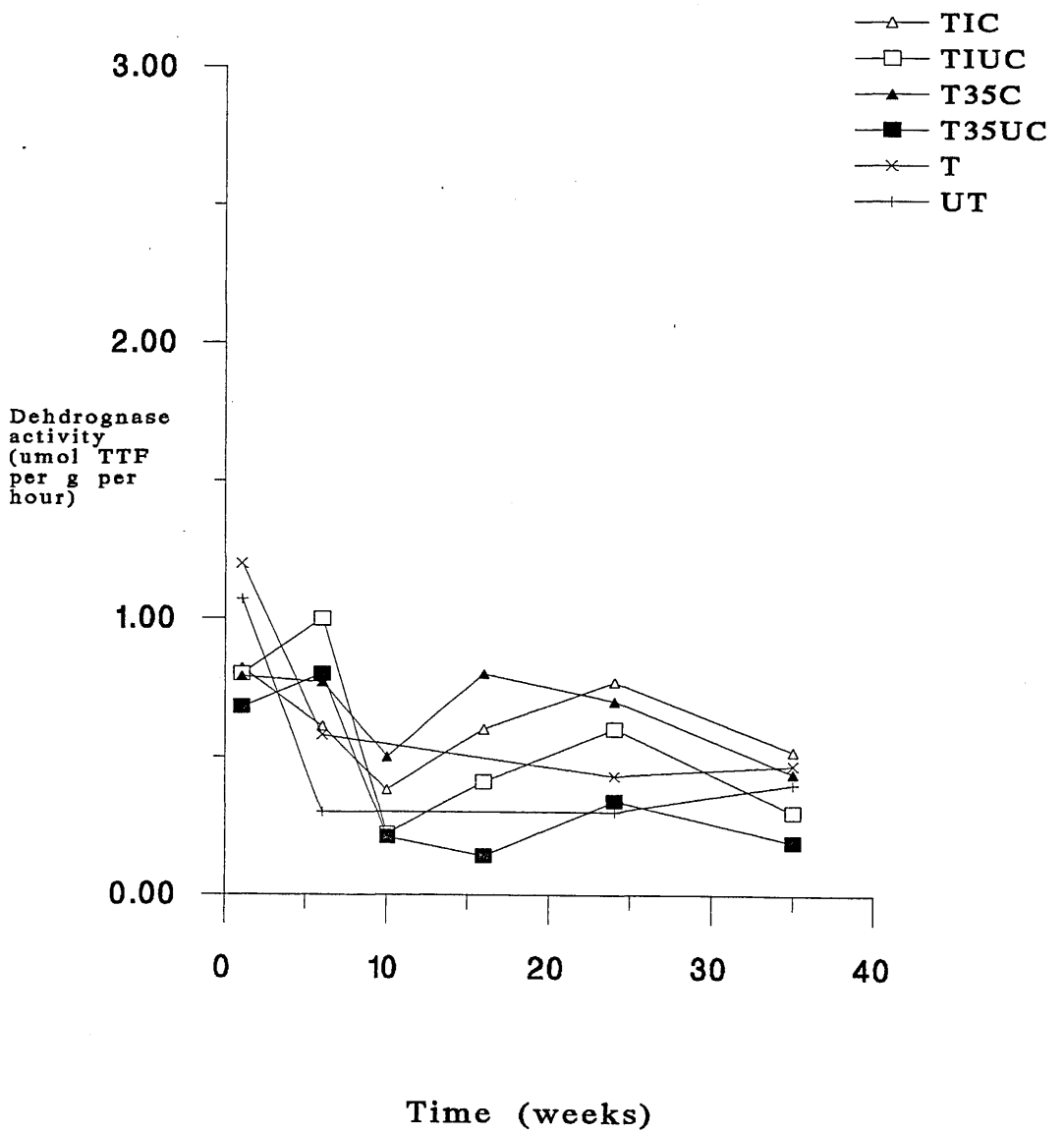


Figure 8.3 Dehydrogenase activity from Sitka spruce sapwood samples. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. T1C represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1. TIUC represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1. T35C represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70. T35UC represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70. T represents control pole sections that were treated with anti-blu prior to seasoning. UT represents control pole sections that were left untreated prior and during seasoning

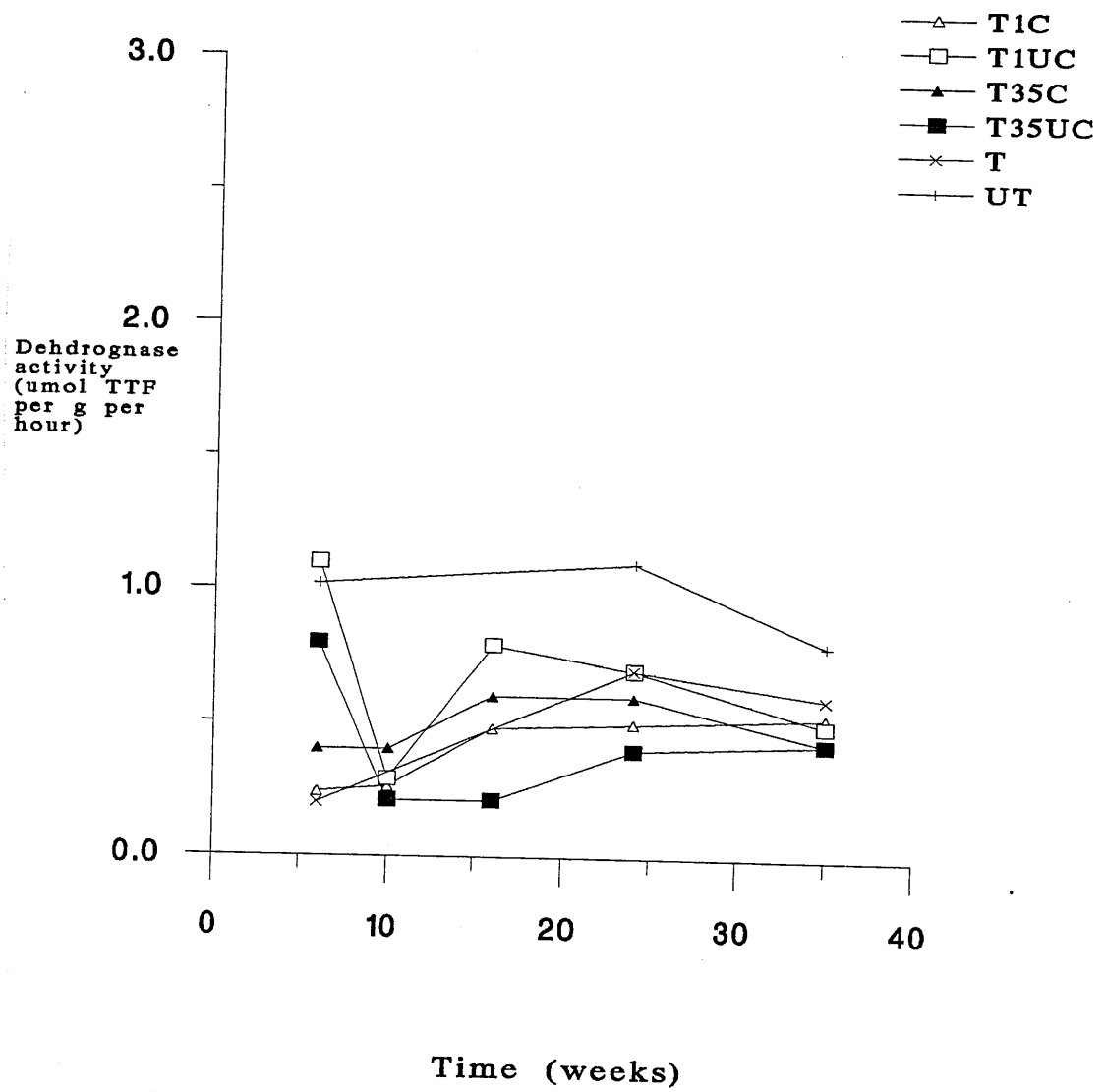


Figure 8.4. Dehydrogenase activity from Sitka spruce heartwood. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. T1C represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1. TIUC represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1. T35C represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70. T35UC represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70. T represents control pole sections that were treated with anti-blu prior to seasoning. UT represents control pole sections that were left untreated prior and during seasoning.

Spruce samples

Dehydrogenase activity in spruce samples showed a general trend which is similar to that recorded in the pine samples although the sapwood values are lower than those recorded in the pine. The activity of the covered samples is again generally higher than the uncovered or control material. This material showed initially high dehydrogenase activity declining after nine weeks followed by a gradual increase for a further 20 weeks before falling throughout the remaining seasoning period. It is noticeable that the two control treatments fell to very low levels of activity after the initial two weeks incubation. The anti blu treated material showed very little residual activity thereafter.

It is also noticeable that for both spruce and pine material large deviations exist in the data making worthwhile interpretations from the results difficult. (See appendix 5)

8.3.3 Moisture Content

Scots pine material

The moisture contents of the logs were measured on a dry weight basis throughout the experiment. The results of these determinations for Scots pine sapwood can be seen in figure 8.5.

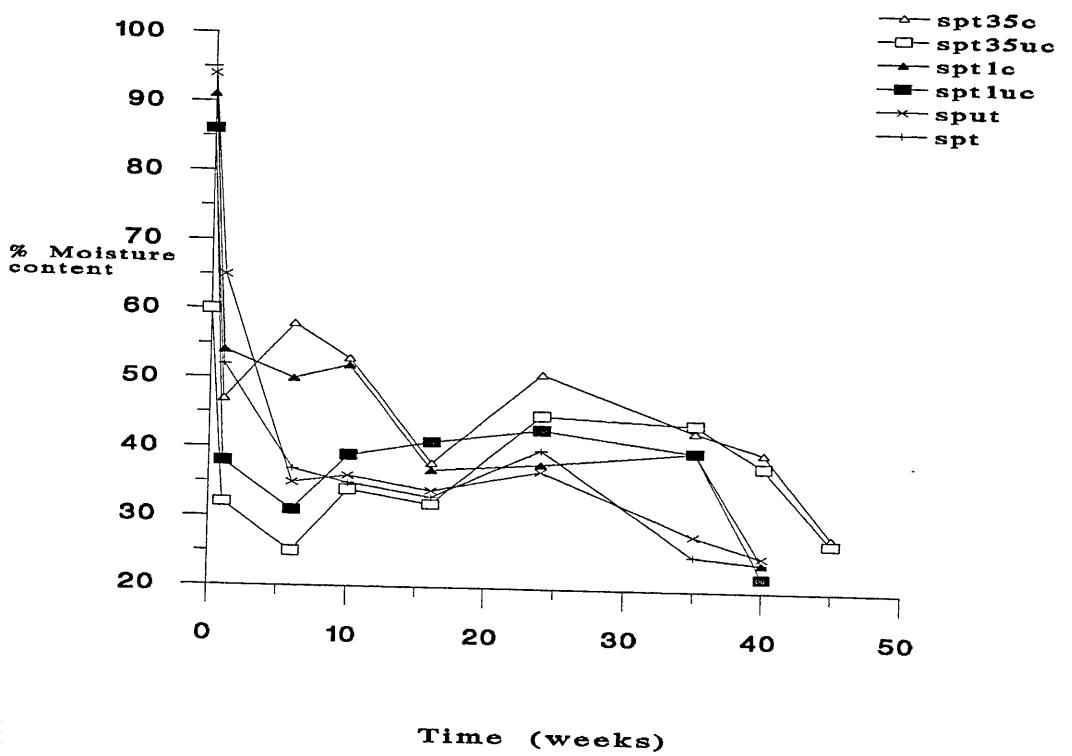


Figure 8.5. Mean % moisture content for sapwood of Scots pine pole sections. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. **spt35c** represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70; **spt35uc** represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70; **spt1c** represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1; spt1uc represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1; **sput** represents control pole sections that were left untreated prior and during seasoning; **spt** represents control pole sections that were treated with anti-blu prior to seasoning.

The results for all pole sections indicate that there was a significant drop in moisture content over the first few days after colonisation. After this initial fall the pine sapwood moisture contents appear almost constant in covered material until the tarpaulin was removed after 10 weeks. The moisture content of the material then fluctuates with the weather conditions until placed undercover at Leven (week 26) when the moisture contents fell below saturation point thereby allowing creosote treatment to proceed. The uncovered samples showed similar fluctuations until covered and then after removal of the tarpaulins the moisture content again fluctuated prior to the 26 week period when stored undercover prior to creosote treatment.

The moisture contents of the control logs showed less variation in moisture content whilst in the field which may be as a consequence of having no *Trichoderma* present and therefore lower permeabilities.

The moisture content of the pine heartwood material can be seen in figure 8.6. Samples of pine heartwood showed a rapid decline in moisture to a level at or below saturation point throughout most of the experiment.

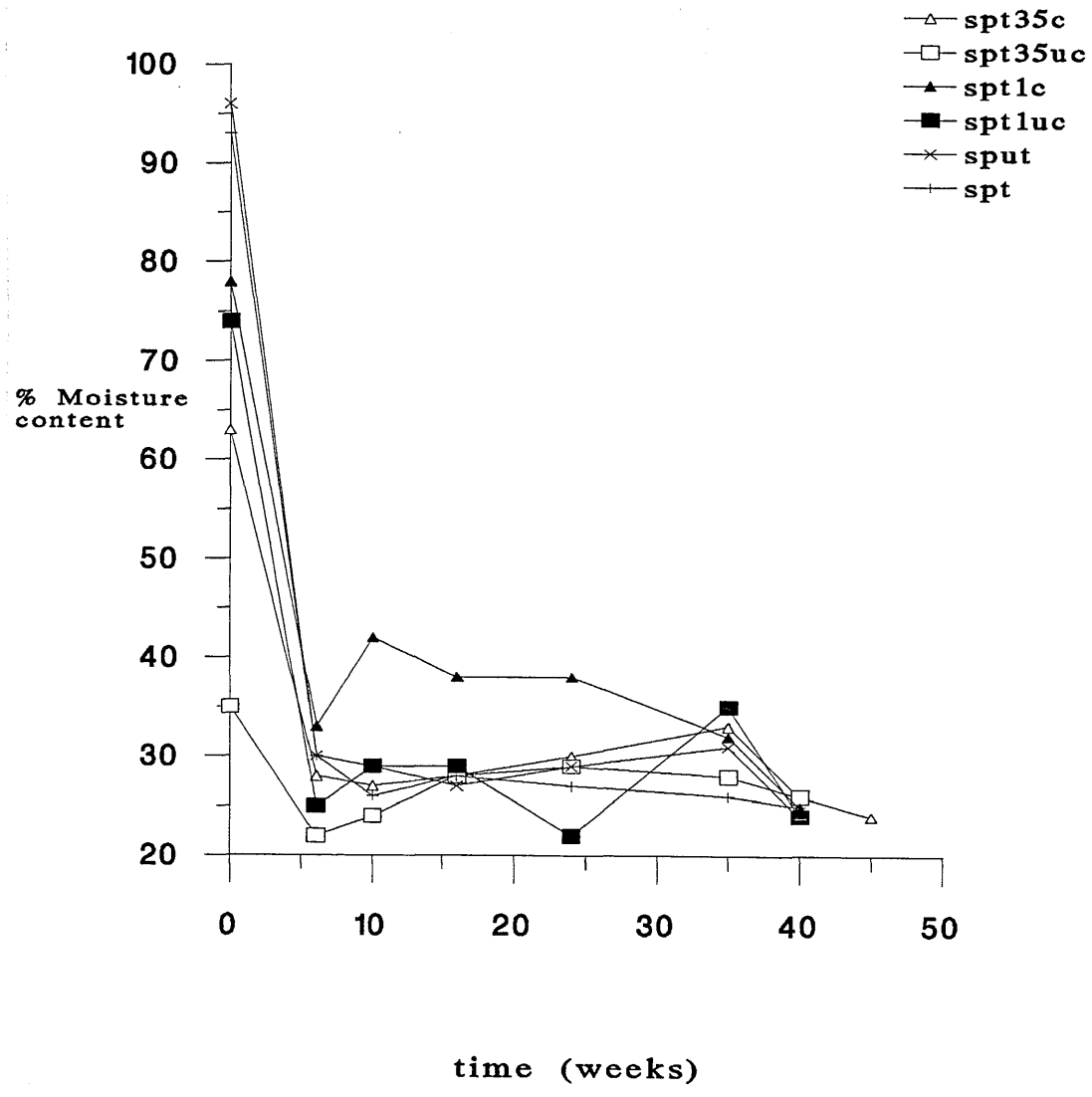


Figure 8.6 Moisture content of pine heartwood samples removed from sample pole sections during incubation and storage. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. **spt35c** represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70; **spt35uc** represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70; **spt1c** represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1; spt1uc represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1; **sput** represents control pole sections that were left untreated prior and during seasoning; **spt** represents control pole sections that were treated with anti-blu prior to seasoning.

Sitka spruce material.

The results of the moisture determinations for Sitka spruce sapwood and heartwood material can be seen in figures 8.7 and 8.8

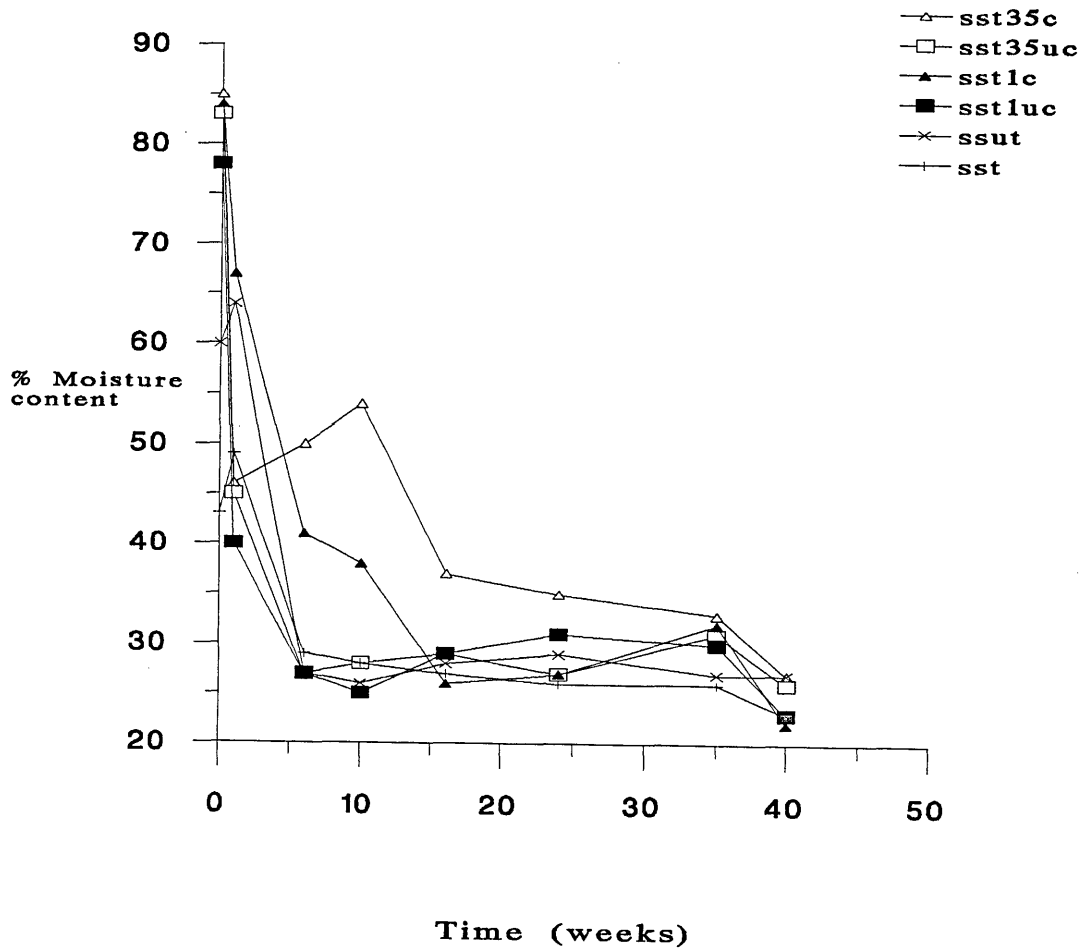


Figure 8.7 Moisture content of spruce sapwood samples removed from sample pole sections. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. **sst35c** represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70; **sst35uc** represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70; **sst1c** represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1; spt1uc represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1; **ssut** represents control pole sections that were left untreated prior and during seasoning; **sst** represents control pole sections that were treated with anti-blu prior to seasoning.

The covered Sitka spruce material had a higher moisture content than the uncovered material in the early stages of the experiment and this would undoubtedly lead to better colonisation of the material by the *Trichoderma* isolates used. When the uncovered material was covered by the tarpaulins the moisture contents of these logs did not rise to the same extent as in the corresponding pine logs. This lack of re-wetting is likely to be due to irreversible drying of the timber and is likely to be linked to the changed permeability of the timber. The timber may be dried before the *Trichoderma* isolates can prevent aspiration and hence the timber is less permeable and may not absorb moisture as readily as more permeable samples. The uncovered material shows a similar profile to those of the control and Anti-blu treated logs.

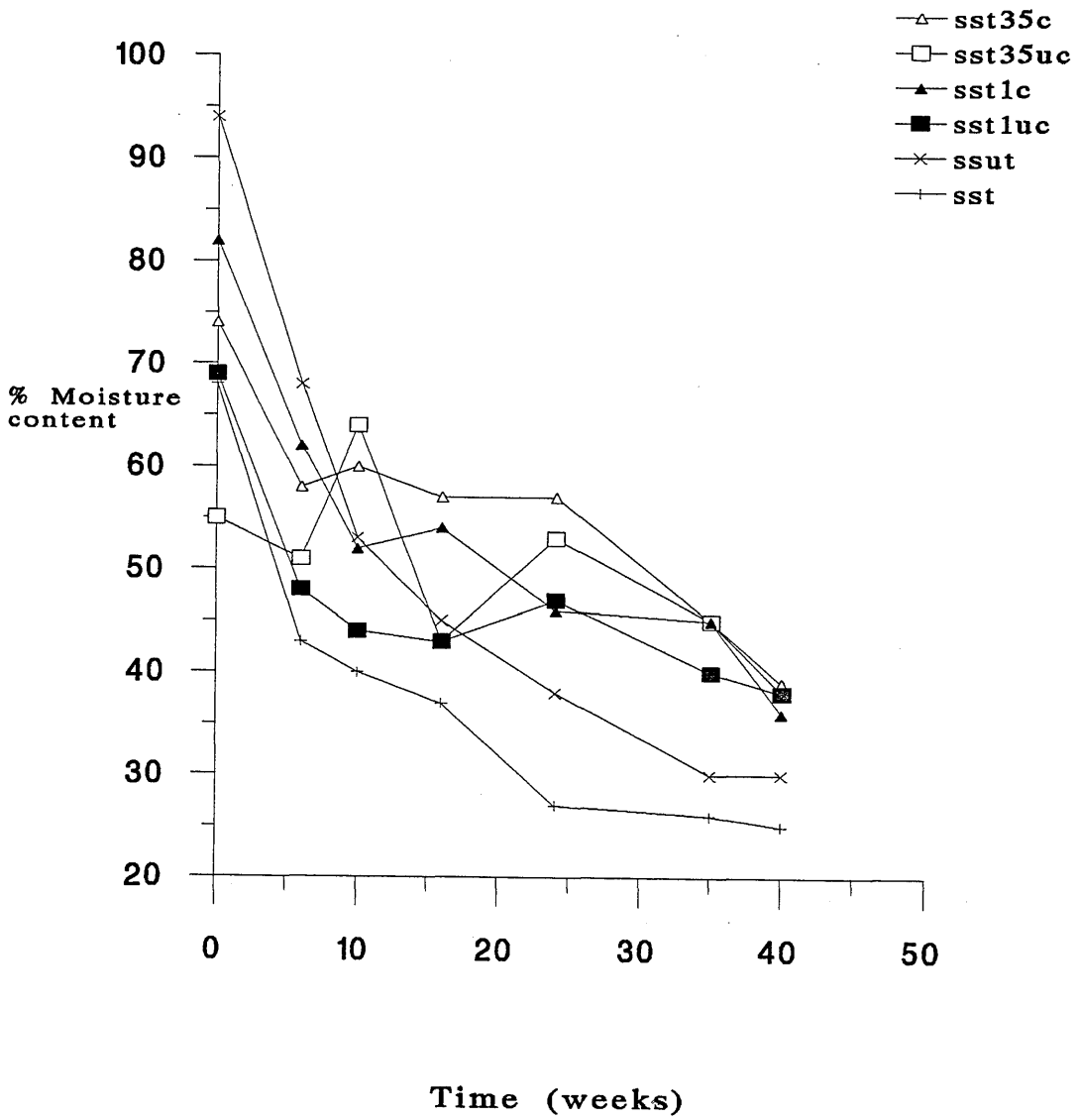


Figure 8.8 Moisture content of spruce heartwood samples removed from sample pole sections. N.B. Error bars have been omitted from this figure as they seriously distracted from the interpretation of the data. NB. **sst35c** represents pole sections which were initially covered and inoculated with *Trichoderma viride* SIWT 70; **sst35uc** represents pole sections which were initially uncovered and inoculated with *Trichoderma viride* SIWT70; **sst1c** represents pole sections which were initially covered and inoculated with *Trichoderma aureoviride* SIWT1; spt1uc represents pole sections which were initially uncovered and inoculated with *Trichoderma aureoviride* SIWT1; **ssut** represents control pole sections that were left untreated prior and during seasoning; **sst** represents control pole sections that were treated with anti-blu prior to seasoning.

The moisture content of spruce heartwood is generally much higher than that observed for pine heartwood and for that recorded for Sitka spruce sapwood. The results shown in figure 8.8 generally show a gradual reduction in the moisture content of the wood samples from this region with time.

8.3.4 Permeability

A summary of the results of the permeability determinations for the sample pole sections can be seen in table 8.3.

	Wood species	
Pole Section Treatment	SITKA SPRUCE Mean % increase (St dev)	SCOTS PINE Mean % increase (St dev)
Anti blu Treated	170 (190)	1735 (680)
Untreated	325 (400)	2090 (1427)
Covered *T. aureoviride*	690 (855)	2830 (3060)
Uncovered *T. aureoviride*	555 (775)	1580 (2320)
Covered *T. viride*	1010 (1480)	3020 (2690)
Uncovered *T. viride*	220 (280)	1515 (1050)

Table 8.3. Mean % increases in permeability of core samples removed from pole sections after seasoning compared with cores removed prior to seasoning. (Six comparisons of cores per log, and 3 logs per treatment).

The results in table 8.3 show that in all treatments there was a mean increase in permeability. The magnitude of this change in permeability varied between treatments and wood types. Generally those logs that had been initially covered at the start of the experiment gave rise to the greatest increases in permeability and those logs treated with Anti Blu showed the lowest increases in permeability, this may have been due to a

reduction in the growth of naturally colonising organisms that will also alter the permeability of the timbers.

It is clear that large standard deviations exist in the increases in permeability data. Each result in table 8.3 represents the mean of 18 values, however it should be noted that this combines the data from 3 pole sections, sampled at 3 separate locations per pole and two sapwood sample depths. The intra- and inter-tree variability in permeability has already been highlighted (Chapter 5) and so it is therefore not entirely unexpected to see the degree of variability recorded here. Because of these deviations, when analysed statistically the results show that there is no significant difference between the treatments.

As with earlier experiments spruce material showed the lowest actual permeability. Pole sections treated with *Trichoderma* generally gave higher mean % increases in permeability with the pole sections that were initially covered at the start of the experiment showing the greatest increases in permeability. The exception to this was the uncovered *T. viride* samples which only showed increases similar to those observed for the control material.

Increases in pine material were greater than those observed in the spruce material, with all sample logs showing an increase in permeability. Pole sections that were initially covered again showed the greatest increases in permeability compared with the other treatments.

8.3.5 Preservative Penetration

After creosote treatment the penetration of preservative into the pole sections was measured. The results for Sitka spruce (Table 8.4) were analysed using the general linear model (GLM) and the results of this analysis indicated that there was no significant difference between the covered and uncovered material. This is probably due to the necessary covering of the previously uncovered material to promote *Trichoderma* colonisation in the early stages of the experiment.

There was however a significant increase in the depth of preservative penetration into Sitka spruce after treatment with *Trichoderma aureoviride* (SIWT1) isolate compared with the untreated log material.

Treatment	No. of samples	Mean penetration (mm)	Standard Deviation
Trichoderma aureoviride (SIWT1)	200	15.5	6.4
Trichoderma viride (SIWT70)	200	12.0	5.7
Anti-blu treated logs	100	8.8	4.1
Untreated logs	100	12.9	4.9

Table 8.4. Creosote penetration into Sitka spruce.
(Note: The results for the covered and uncovered *Trichoderma* isolates were pooled as no significant difference was found between the two treatments.)

The results show that there is a significant increase in penetration into spruce pole sections that have been pre-treated with *Trichoderma aureoviride* (SIWT1). The *Trichoderma viride* (SIWT70) treated pole sections showed similar depths of preservative penetration to untreated logs. The logs treated with anti-blu showed the lowest penetration of the creosoted logs and were significantly lower than the other treatments, indicating that the anti-stain treatment inhibited the natural colonisers that would naturally help to increase preservative penetration. None of the spruce sections treated with creosote showed full sapwood penetration when depth of creosote penetration was compared with sapwood depth when stained with Bromo creosol green (which showed a mean depth of 45mm). The results of preservative penetration into pine pole sections showed no significant increases in depth of penetration after treatment with *Trichoderma* isolates. All of the sampled sections however showed full sapwood penetration and the lack of significant increases in permeability reflects the natural treatability of pine.

8.3.6 Preservative Loadings

Preservative retention in the creosote treated region of spruce sapwood of control and *Trichoderma* treated pole sections can be seen in figure 8.9.

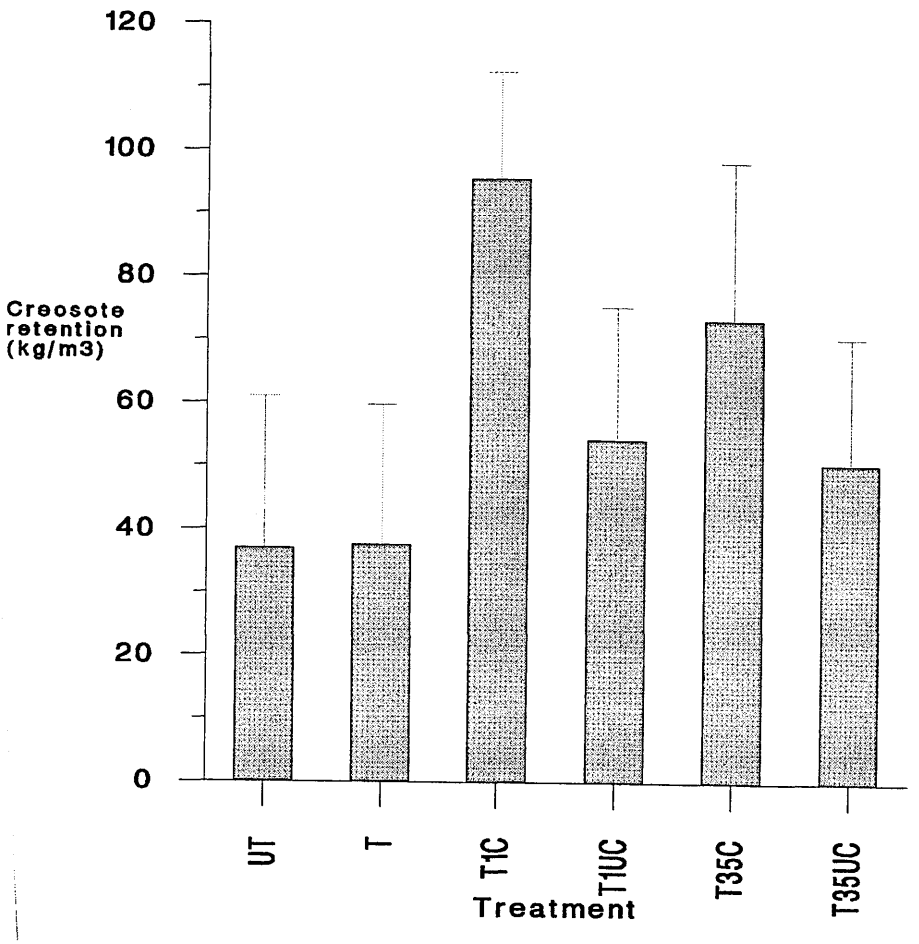


Figure 8.9- Mean creosote retention (kg/m^3) in the treated sapwood region of Sitka spruce poles. N.B Error bars were calculated as the standard deviation of the data set; T represents control pole sections treated with an anti-sap stain chemical; UT represents untreated control pole sections; T1C represents sections treated with *Trichoderma aureoviride* (SIWT1) and initially covered during incubation; T1UC represents pole sections treated with *Trichoderma aureoviride* (SIWT1) and uncovered during incubation; T35C represents pole sections treated with *Trichoderma viride* (SIWT70) and initially covered during incubation; T35UC represents pole sections treated with *Trichoderma viride* (SIWT70) and uncovered during incubation. (Each value represents the mean of 20 cores pooled for each log, the results of which were combined for each treatment group)

Sections inoculated with *Trichoderma aureoviride* showed the highest penetration (table 8.4 and retention (almost 3 times that of the control sections) compared with the other pole material. The two control treatments showed similar levels of loading despite there being significant differences in the penetration depth of the preservative into the sections.

The creosote retention in the entire spruce sapwood can be seen in Figure 8.10.

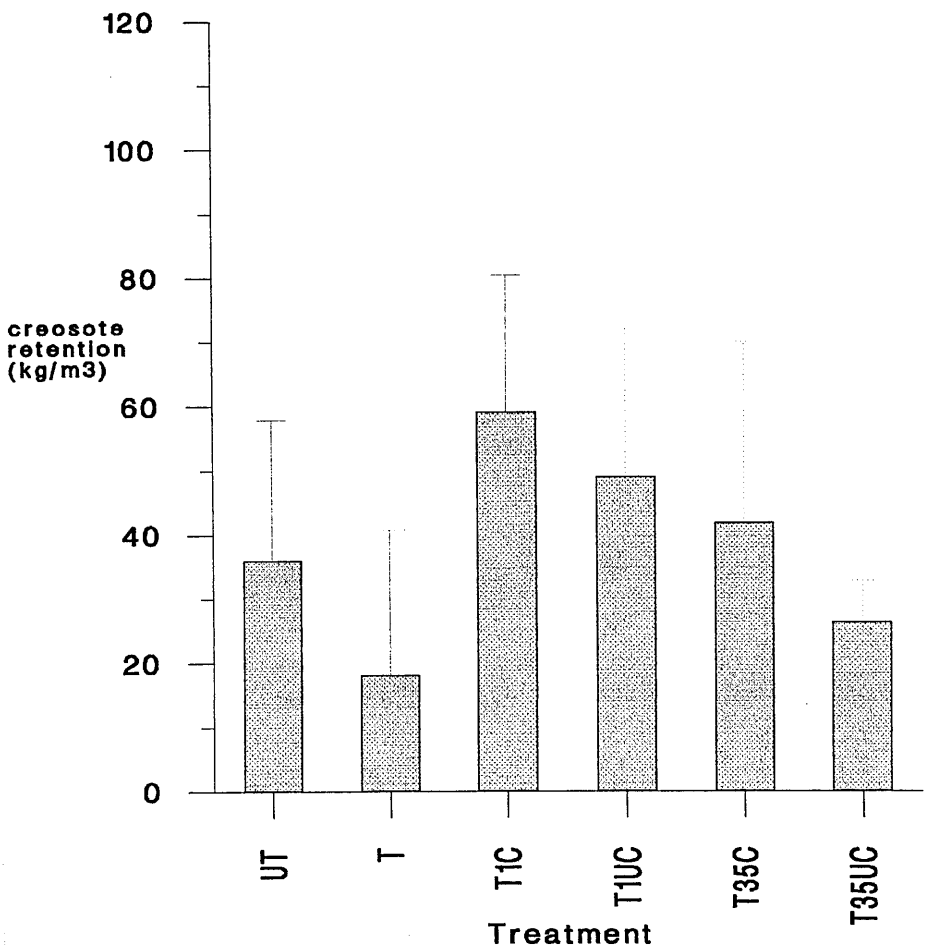


Figure 8.10. Mean creosote retention (kg/m^3) in the entire sapwood region of Sitka spruce poles. N.B Error bars were calculated as the standard deviation of the data set; T represents control pole sections treated with an anti-sap stain chemical; UT represents untreated control pole sections; T1C represents sections treated with *Trichoderma aureoviride* (SIWT1) and initially covered during incubation; T1UC represents pole sections treated with *Trichoderma aureoviride* (SIWT1) and uncovered during incubation; T35C represents pole sections treated with *Trichoderma viride* (SIWT70) and initially covered during incubation; T35UC represents pole sections treated with *Trichoderma viride* (SIWT70) and uncovered during incubation.

These results show that again the highest retentions were with the *Trichoderma aureoviride* (SIWT1) treated pole material. The pole sections that had been treated with the anti-blu preservative prior to the experiment showed the lowest preservative retention and this illustrates the poor penetration observed with this treatment (table 8.4). *Trichoderma aureoviride* (SIWT1) significantly improved the penetration and retention of preservative in the pole material, however it should be noted that in both determinations the sampled spruce logs failed to reach the required minimum retention of 115 kg/m^3 and full sapwood penetration as detailed in ESI 43-88 (1987). This figure represents a retention of 115 kg/m^3 throughout the cross section of the pole including the heartwood. The results reported here for spruce represent only loadings in the sapwood. It is clear from the data for pole diameter and sapwood depth however that even the corrected value for TIC of 27 kg/m^3 (examples of how this is calculated can be seen in appendix 6) is well below the require figure of 115 kg/m^3. Hainey (1992) found similar preservative patterns with the sap displacement of Sitka spruce with CCA, when this preservative gave lower penetration and loadings in the treated zone of spruce compared with Scots pine.

All pine pole loadings were assessed in the treated zone at varying depths (every 10mm) from the surface. In spruce however since full sapwood penetration was not achieved loadings could only be recorded for both the treated zone and the entire sapwood. The results for the preservative retentions in the Scots pine material can be seen in figures 8.11 and 8.12.

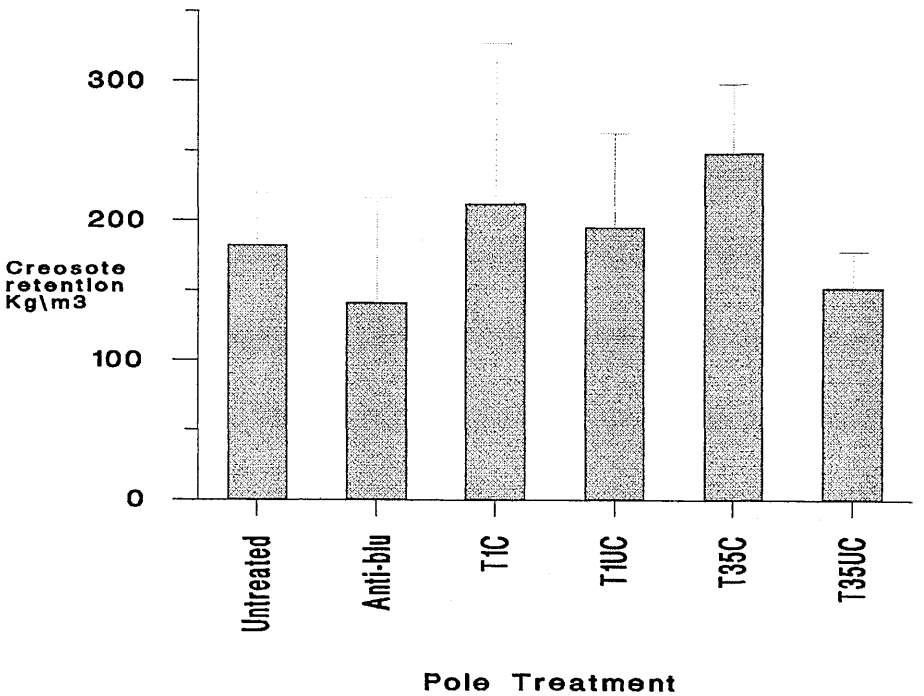


Figure 8.11- Mean creosote retention (kg/m^3) in the preservative treated region of Scots pine pole material. N.B Error bars were calculated as the standard deviation of the data set; Anti-blu represents control pole sections treated with an anti-sap stain chemical; Untreated represents untreated control pole sections; T1C represents sections treated with *Trichoderma aureoviride* (SIWT1) and initially covered during incubation; T1UC represents pole sections treated with *Trichoderma aureoviride* (SIWT1) and uncovered during incubation; T35C represents pole sections treated with *Trichoderma viride* (SIWT70) and initially covered during incubation; T35UC represents pole sections treated with *Trichoderma viride* (SIWT70) and uncovered during incubation.

The results in figure 8.11, show highest retentions with the pole sections that were initially covered and treated with *Trichoderma viride* (SIWT70). The preservative retentions in the creosoted pole sections indicate that in all instances full sapwood penetration was achieved and in all treatments except some anti-blu treated controls the minimum retention exceeded the required 115 kg/m^3 (ESI 43-88, 1987). The radial distribution of preservative in the Scots pine poles can be seen in Figure 8.12.

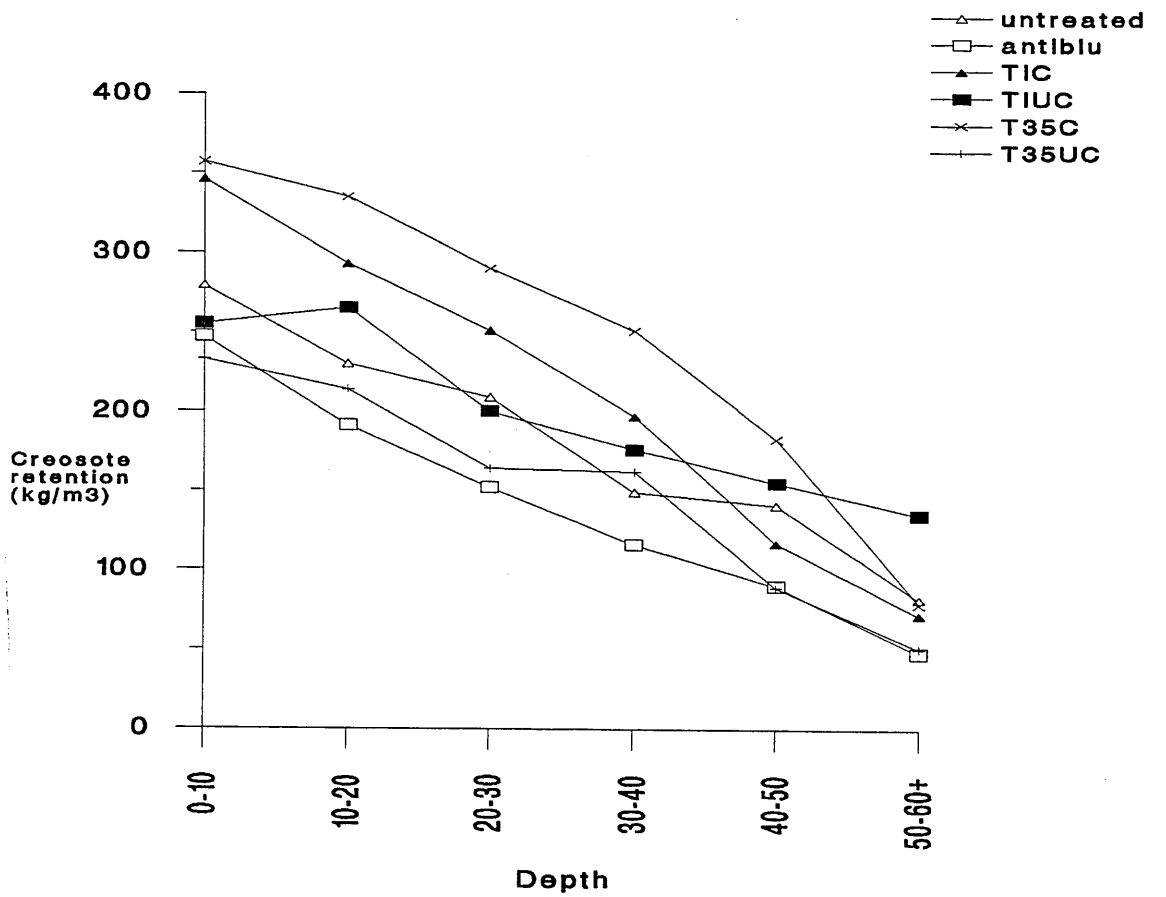


Figure 8.12. Retention of creosote (kg/m^3) in different regions of the Scots pine poles. N.B Error bars are omitted as they seriously detracted from the clarity of the figure; Anti-blu represents control pole sections treated with an anti-sap stain chemical; Untreated represents untreated control pole sections; T1C represents sections treated with *Trichoderma aureoviride* (SIWT1) and initially covered during incubation; T1UC represents pole sections treated with *Trichoderma aureoviride* (SIWT1) and uncovered during incubation; T35C represents pole sections treated with *Trichoderma viride*

(SIWT70) and initially covered during incubation; T35UC represents pole sections treated with *Trichoderma viride* (SIWT70) and uncovered during incubation.

The results indicate that the covered pole sections treated with *Trichoderma viride* (SIWT70) and *Trichoderma aureoviride* (SIWT1) showed the best retentions particularly in the outer layers of the sapwood where loadings were considerably in excess of the minimum retention required for protection. Higher levels in the surface layers of the poles is valuable for improved protection primarily against soft rot fungi. It may be, however, that these increased loadings could result in increased bleeding from the poles although it should be noted that these poles appeared as dry as the control material on removal from the treatment cylinder. The lowest retention for the treatments was again observed in the anti-blu treated material, indicating that natural microflora induced increases in preservative penetration may be prevented by pretreatment with this preservative. Prior to the preservative treatment the heartwood region of the pine poles was measured in four compass directions (north, south, east and west). After preservative treatment the penetration of the creosote was again measured. A comparison with earlier measurements for the logs showed that the preservative penetration into pine heartwood was not significantly increased after treatment with *Trichoderma aureoviride* (SIWT1) but preservative did penetrate the outer heartwood region (2-3mm) of all pine logs including controls.

8.3.7 *Trichoderma* isolation after creosote treatment

Cores removed from the logs for the purpose of isolation were aseptically plated out on malt extract agar and any resulting growth noted. The results for Scots pine and Sitka spruce pole sections can be seen in tables 8.5 and 8.6.

Isolations of *Trichoderma* from core removed from Scot pine logs can be seen in table 8.5.

Wood species	Treatment	No. cores	No.of *Trichoderma* Isolations
Scots pine	*T.aureoviride* covered	10	0
	T.aureoviride uncovered	10	2
	T.viride covered	10	0
	T.viride uncovered	10	0
	Anti blu treated	10	0
	control	10	0

Table 8.5. Isolations of *Trichoderma* from pine pole sections after treatment with creosote.

Wood species	Treatment	No. cores	No.of *Trichoderma* Isolations
Sitka spruce	*T. aureoviride* covered	10	8
	T. aureoviride uncovered	10	8
	T. viride covered	10	4
	T. viride uncovered	10	6
	Anti blu treated	10	1
	control	10	0

Table 8.6. Isolations of *Trichoderma* from spruce pole sections after treatment with creosote.

No *Trichoderma* was isolated from the preservative treated zones of either Scots pine or Sitka spruce. It is clear that in most pine samples the *Trichoderma* isolates were unable to

survive the preservative treatment process although 2 isolates of *Trichoderma aureoviride* SIWT1 were removed from the heartwood of the uncovered Scots pine. However this was not generally the case with the spruce material as up to 80% of the logs sampled for each treatment showed growth of *Trichoderma* after preservative treatment. This clearly indicates that the temperature reached within the wood during treatment is not high enough to sterilize the wood and that pretreatment colonisers including decay fungi may survive the treatment process. It is interesting to note the presence of a *Trichoderma* coloniser in one of the anti-blu treated samples.

8.4 Discussion

Initial colonisation results indicated that the colonisation of the covered material was good and that the uncovered material showed little establishment of *Trichoderma*. After remedial treatment of uncovered material, subsequent isolations of *Trichoderma* from the pole material proved successful. However it is clear that the moisture content of the uncovered material dropped significantly with time (figures 8.5-9) and was partially responsible for the initial lack of *Trichoderma* growth from the pole sections. If this process is to be used commercially then the problems experienced with initial colonisation would have to be addressed further. Raising the humidity around the pole sections led to successful colonisation. The fact that *Trichoderma* inoculated onto material which was initially uncovered struggled to establish and subsequent preservative treatment was not as effective indicates that efficient establishment of the organism is of primary importance to the success of the treatment. Given the weather experienced in the UK it is likely that special treatments of the wood products will have to be developed to ensure good colonisation by the organism.

Dehydrogenase activity showed a general decline with time indicating that the microbial activity within the logs was dropping over this period. The microbial activity as measured through the dehydrogenase assay appeared to be greater during periods where higher

moisture contents were observed. This may imply that the conditions within the wood were not conducive to growth and the addition of more moisture permitted better growth of the organisms in the wood. Rain water is not sterile and in the wetting up of the logs other organisms may also be deposited which may increase microbial activity. It is obvious from the dehydrogenase results that there were large fluctuations in the results at various time intervals and also large inter-log variations. Since this was a field test system some of these fluctuations are likely to be linked to the weather conditions experienced where daily variations in temperature and humidity are likely to vary the growth of the isolates. The dehydrogenase assay is a measure of the production of this enzyme during the growth of the organism. This is an indicator of the biological activity of organisms in samples. Hainey (1992) used this method to determine biological activity in soil adjacent to buried wood samples. Her results showed raised activity in these sites compared to background samples. These experiments were however conducted in a constant environment unlike the present study. The sampling periods used in this study are unlikely to detect such subtle changes in the growth/ activity of the organisms within the wood samples.

Moisture contents were measured throughout the experiment and the results showed that while the logs were covered the moisture content remained relatively constant but after the logs were uncovered the moisture contents fluctuated as the timber dried down and wet up again as it absorbed rain water. Spruce logs treated with *Trichoderma aureoviride* (SIWT1) show a higher moisture content (at 25 weeks) in the sapwood after a prolonged period of heavy rain compared with spruce controls. This may indicate that the treatment is increasing the rate of moisture uptake due to increased permeability caused by the *Trichoderma* treatment. Spruce heartwood consistently showed higher moistures than those observed in the sapwood material. This is likely to be due to the way in which spruce dries. Bordered pits will aspirate at the point of air moisture interface and will subsequently collapse as further drying occurs (Saiu, 1984). This process moves a drying front in to the wood and will maintain moisture levels within the timber. Pine does not

exhibit the same type of drying regime and heartwood material is generally lower in moisture than the spruce equivalent (Fakhouri *et al*, 1993).

Pine logs treated with *Trichoderma viride* (SIWT70) also showed an increase in moisture over the same time period.

The pole sections treated with the selected *Trichoderma* isolates did not show the same magnitude of permeability increases as exhibited in the small roundwood log samples (Chapter 5). This is likely to be due to the different incubation regimes used in the experiments. With the smaller material it was possible to incubate the logs completely covered in plastic bags under more regulated temperature conditions. This was however impractical for the larger material. Less moisture was also lost from the smaller logs and hence it is likely that colonisation was better for the small log samples than was observed in pole sections which in turn would lead to improvement in the permeability to a much greater extent. The pole sections also suffered from the exceptional weather experienced during the initial and final stages of the experiment. The inoculation and initial incubation was done whilst the UK was experiencing one the driest summers (1994) on record. As the timber samples dried out pit aspiration would occur and this could delay colonisation and make subsequent spraying to increase moisture content more inefficient. This and the treatment delays experienced prior to inoculation would contribute to some of the problems experienced in the establishment of the organisms within the timber and could subsequently affect permeability enhancement.

Despite this the permeability of *Trichoderma* treated pole sections still showed greater increases than control sections. Pole material that was covered in the early stages of the experiment showed better increases in permeability than either the control or uncovered material. This improvement in permeability was likely to be due to better colonisation of the covered material and was responsible for the better preservative penetration observed in the *Trichoderma* treated timbers.

From the results in table 8.4 it can be seen that the penetration of preservative in the spruce material has been improved with incubation of the pole sections with *Trichoderma.* The results show a significant increase in penetration in pole sections that are treated with *Trichoderma aureoviride* SIWT 1. The analysis of penetration however showed no significant differences between the covered and uncovered pole sections but those treated with *Trichoderma aureoviride* SIWT 1 did show significant increases in penetration compared with both control materials i.e. both Anti-blu and untreated. The use of the Anti-blu on the timber would prevent sapstain and other contaminant organisms from growing through the material. Any increase in penetration compared to this material must therefore be due to the action of biological agents. The untreated control material will indicate the penetration expected if spruce material was treated in a manner similar to that currently used for pole production. Hence from these results it is expected that *Trichoderma* treatment will improve the penetration of creosote into the treated material. All of the pine material sampled showed full sapwood penetration and from measurements made prior to the preservative treatment some penetration of preservative was observed into the heartwood region. This however was not dependent on the whether the pole section had been treated with *Trichoderma* as the control samples displayed the same effect.

Despite improved penetration of preservative into spruce material after *Trichoderma* treatment it is unlikely that the improvements observed here would significantly alter the service life of the timber. This is due to the depth of penetration being less than the expected depth of checks that will develop in the timber. If checks extend beyond the treated zone then it is likely that decay organisms will be capable of establishing within the timber.

The preservative loadings at increasing 10mm depths were measured for pine samples only, as the spruce material rarely gave large enough or deeply penetrated samples to carry out a meaningful determination. The pine material showed some interesting results as generally the outer 10mm showed the highest levels of preservative, as the depth within

the pole section was increased the amount of preservative observed declined. Similar profiles were observed by Hainey (1992) where the concentration of CCA declined with depth into the timber. The covered material showed the highest levels of preservative in the outer regions. This went against an initial hypothesis that increasing the permeability of the wood material may in fact reduce the amount of preservative left in the surface regions after treatment as more preservative would be removed by the final treating vacuum. This however appears not to be the case. The more permeable regions may absorb more preservative by simple infusion after the final vacuum is pulled. It may also be possible that increasing the permeability will allow a more complete covering of the wood cell walls by the preservative during treatment and hence a higher surface area being treated with creosote resulting in greater retentions.

Results of the total preservative loadings in treated regions for both timber species show that more preservative is observed in samples pre-treated with the selected *Trichoderma* isolates. Since increases in permeability are observed in timbers treated with *Trichoderma* it is reasonable to assume that the increases in preservative loadings and penetration in pole sections is due to the same action of the selected isolates.

The largest amounts of preservative were observed in the covered pine samples which also showed the highest amounts in the outer regions. This extra preservative in the outer regions may be beneficial in regions where the poles are likely to be subjected to soft rot as the higher surface level would give better protection from these organisms.

In the spruce material the highest loadings were also observed in the *Trichoderma* treated sections. These results showed not only increased amounts but deeper penetration of preservative.

All pine pole sections showed adequate penetration and loading to meet the standard requirements for the preservation of pole material. However the spruce material even after *Trichoderma* treatment still remains below the levels required by ESI 43-88 (1987). The poor penetration in the spruce material may be partially due to the preservative treatment regime used. Under normal circumstances the preservation of spruce material is by double

vacuum (BS 1990, 1984), but in this experiment the empty cell process was used. The fact that the treatment of these poles sections with *Trichoderma* resulted in an increase in permeability and higher preservative loadings than in control material indicates that this process does work.

If the experiment was repeated then improvements would be made in the methods used to establish the organisms within the timber. A faster inoculation method would be used i.e. a dipping system and this would be done immediately after debarking. The timber would be covered to raise the humidity around the logs to encourage more efficient colonisation. On completion of the experiment if a double vacuum process is used to treat the spruce material it is likely that the penetration and loadings in the timber can be increased significantly.

After the preservative treatment the cores removed from the logs for microbial analysis showed some interesting results. The pine logs showed little or no growth from the cores, this is not surprising given the levels of creosote observed in the core sections close to the heartwood centre of the pole. However the spruce material showed some surprising results, as the majority of cores removed from the *Trichoderma* treated logs showed evidence of microbial growth with *Trichoderma* isolates growing from the untreated regions i.e. inner sapwood and heartwood. If *Trichoderma* can survive the treatment process, other organisms may also be able to survive the treatment and if these are decay fungi, degrade the timber in service. The isolates used in this experiment have also been shown to have some biological control abilities (Srinivasan, 1993) and it may be possible that in the future integrated (chemical and biological) control systems could be used to protect timber that is difficult to treat. With these *Trichoderma* isolates surviving in the treated timber it may be possible that they could prevent the on-set of internal decay by other organisms and protect the timber in areas that are not or cannot be reached by the preservative even after permeability enhancement.

Chapter 9 General Discussion

The use of biological agents to improve the permeability of timber has been reported by a number of authors (Chidester, 1942; Lindgren and Harvey, 1952; Graham, 1954; Lindgren & Wright, 1954; Schulz, 1956; De Freitas and Erickson, 1969; Johnson & Gjovik 1970; Dunleavy and McQuire, 1970; Bauch *et al*, 1970; Dunleavy and Fogarty, 1971: Fowlie and Sheard, 1983). In all cases there was an increase in permeability of the timber and where tested improvements in the absorption of fluids (preservatives) into the timber.

Ponding of timber has previously shown some of the best improvements in Spruce permeability with the process being introduced commercially for a period in Ireland (Fowlie and Sheard, 1983). However given the drawbacks associated with this process and the financial penalties that extended seasoning impose it is likely that the use of biological agents that do not substantially increase the seasoning time would be commercially advantageous. Ponding by its nature will ensure that the timber is saturated until removed from the water and the time required to season the timber will be extended. Financially the longer a piece of timber takes to season the greater the time taken to realise a return on the timber and the greater the financial penalty for using this treatment process. Results from this study indicate that permeability enhancement can be achieved using selected *Trichoderma* agents and with little or no adverse effect on the subsequent seasoning period required prior to preservative treatment.

The use of agents like *Trichoderma* to improve the permeability of the timber has the obvious advantage that the timber does not have to be submerged for several months. This study has shown that once selected *Trichoderma* isolates are established in timber they will grow effectively through the wood and significantly increase the permeability of the timber.

The field trial highlighted some of the problems that may be experienced in the establishment of the *Trichoderma* isolates into pole material. Where timber dried out prior to and just after inoculation, poor colonisation was observed. The use of tarpaulins to raise the humidity next to the timber increased the effectiveness and rate of colonisation by the isolates. Colonisation by the *Trichoderma* isolates had significant implications for the subsequent preservative treatment of the timber, with those pole sections that were covered and efficiently colonised showing significant increases in permeability. Material that was initially uncovered and dried out prior to inoculation (aspirated) showed poorer preservative uptakes and penetration on completion of the study. Hence initial colonisation of the timber by the isolates is vital to the final improvements in permeability. *Trichoderma* isolates are the ideal organism for this process as they are pioneer organisms at the start of an ecological successions on disturbed sites (Dowding, 1973; Domsch *et al,* 1980). They can grow rapidly through the timber to the exclusion of other organisms and as this study has shown improve the final permeability of the timber. However if the colonisation process is hindered then the resulting improvements may be reduced. If this process is to be used commercially then it is essential that the problems with colonisation experienced in this study are addressed and that any enhancement in the process should not adversely affect the seasoning time of the timber.

The *Trichoderma* isolates used in this study were selected on their ability to produce enzymes *in vitro.* The use of a medium which had a similar C:N ratio to wood was aimed to model what would happen in the natural material. However given the wide range of C:N ratios that exist in different wood types and that woods also contain different compounds i.e. extractives which may or may not affect the growth and enzyme production by the organisms, the results from media experiments required confirmation from testing in wood samples. This is a limitation with all laboratory tests and models since growth conditions are usually strictly controlled and the influencing factors are managed. The composition of wood as a growth substrate will be dependent on the species of tree encountered and the conditions in which it was grown; the position within

the forest stand and whether it was damaged in any way prior to harvesting. Wood is highly variable with no two trees being the same or even within the timber from one tree no two blocks being exactly the same, and variation will affect the growth of organisms through the timber in different ways. Given this variation it is obvious that laboratory testing will not always be a relevant substitute for tests in wood.

Despite these limitations the enzyme production trials showed the diversity in the ability of different *Trichoderma* isolates to produce the selected enzymes. When the best of the isolates selected on the basis of autecological studies were tested in wood samples it was possible to identify that two isolates continued to show a significant increase in their ability to produce enzymes to improve the permeability of the timber.

Studies into the ability of the isolates to grow in the presence of the water soluble extracts highlighted some interesting results. Although all isolates were inhibited by the presence of these compounds, most would still grow in the presence of some Scots pine heartwood extracts, indicating that these isolates may be able to grow through the pine heartwood region which was previously thought too inhospitable for growth. Further work on wood poles (Chapter 8) confirmed that these isolates were capable of growing through the heartwood regions, this has major two implications:

1) Isolates may be capable of improving the permeability and subsequent preservative treatment of the heartwood and allow effective treatment of this region thus preventing decay organisms from establishing in this region and prolonging the service life of the timber.

2) Since *Trichoderma* are associated with being biological control agents it is possible that by growing through the heartwood region they may remain in this region and protect the timber from subsequent invasion by decay organisms.

Indeed results from this study have shown that in the untreated regions of some of the pole material, *Trichoderma* isolates survived the pressure treatment process and given that one of the isolates (*Trichoderma aureoviride* SIWT 1) used in the field trial had previously shown some biological control capabilities (Srinivasan 1993), it is possible that the treated timber would benefit from the protection given by the presence of these organisms. Obviously further work on decay resistance of such material would be required to establish whether any extra benefit was being derived from the survival of the organisms after treatment. Should this prove to be beneficial then it enhances the possibility of integrated preservative systems where less preservative could be used to treat material or alternatively timber previously unsuited to preservative treatment could be developed for use in new areas where it was combined with such a control agent.

Ultimately industry would prefer to use biological agents that would reduce seasoning times, whilst at the same time improving the quality of the end product. It is debatable whether those companies involved in the preservative treatment of timber would welcome a more permeable product, without the quality of the end product being substantially improved and hence attracting a premium. Two factors would influence this reasoning:

1) More permeable timber would absorb more preservative making the treatment process more expensive.
2) Better treated timber may have a longer service life, causing a drop in future demand for the products.

These are problems for the producers of such improved timber products. End users may prefer to have an extended service life from the better treated products. Distribution poles are expected to be in service for approximately 25 years, if the average service life of the poles could be extended by 3-5 years this could give rise to substantial savings as poles will not need to be replaced as often.

Further work will be required before this process can be used effectively in the industrial sector. No work has been undertaken into the effect of the selected *Trichoderma* isolates

on the strength of the timber after incubation. Since *Trichoderma* are not associated with the production of enzymes capable of breaking down the lignified layers of the wood structure, it is likely that any reduction in strength would be minimal, although obviously the strength issues would have to be addressed prior to this process being used commercially.

Improving the permeability of timber increases the treatability of the wood. If effective preservative treatment can be established for timber species that were previously difficult to treat then new uses for such timber can be established. Timber that is in contact with the ground will be more susceptible to attack from decay organisms e.g. fence posts compared to material that is held out of contact with the ground. At present various softwood timbers are used for these applications and there is no differentiation between timber type or species. If less permeable timber can be treated with preservative and the treatment results in a more effective material then the end product will have a longer service life. However this demand for better timber products will have to be consumer driven as extra treatment will cost the producer in terms of time and raw materials used, increasing the cost of the end product. There is no reason why refractory species that are harder to treat with preservative could not be used for a wider range of products after treatment with *Trichoderma* isolates, except that end users may be discouraged by price. The timber industry in the UK imports the majority of the timber used for poles and other products which is linked to the inability of currently productive forests to supply a quality product at a reasonable price. Distribution poles attract a premium because of the strict criteria that they must meet in terms of size, straightness and number of knots. Being able to use home grown timber more effectively may aid the economy within the UK by reducing imports of timber. Since 90% of Forestry Commission plantings in the UK have been of Sitka spruce it may be an added benefit to be able to use this readily available timber for more than just pulp or carcassing.

Two isolates were used in the field trial in this study. Both carefully selected on the basis of agar and wood block tests to have shown an ability to increase the permeability of Scots pine and Sitka spruce. When the field trials were completed it was seen that *Trichoderma aureoviride* SIWT1 had significantly increased the preservative uptake of Sitka spruce material, whilst the *Trichoderma viride* SIWT 70 showed significant increases in the permeability of Scots pine samples.

Given that these isolates have improved the permeability of the timber species, future work will have to concentrate on making these increases more efficient. Ensuring better colonisation will ultimately result in better preservative treatment. Work will also have to address those areas which were out with the bounds of this study i.e. the effect of the treatment on the strength of treated timber; the effect of the treated material on service life and the environmental impact of preservative from the enhanced treated product to ensure that no unforeseen consequences of the treatment will result.

Conclusions

From this study it is possible to conclude that:

1) *Trichoderma* isolates varied widely in their ability to produce cellulase, pectinase and amylase enzymes on low nutrient medium representative of the C:N balances in wood. The presence of glucose in the growth medium repressed the production of amylase and pectinase in most isolates tested. However despite this repression the isolates selected for further study proved capable of producing amounts of these enzymes in wood, which in turn improved the permeability of the timber.

2) When selected isolates were grown on fresh and dried Scots pine and Sitka spruce sapwood and heartwood, they showed differences in their ability to improve the permeability of the two wood species when treated with organic solvent. Only two isolates consistently showed the ability to improve the permeability in both timber species.

3) When *Trichoderma* isolates were grown on medium containing hot water extracts from Scots pine and Sitka spruce sapwood and heartwood growth inhibition was evident. The levels of inhibition varied between the organisms and was dependent on the type of wood extract. Scots pine heartwood showed the highest inhibition of growth, although *Trichoderma* isolates were still able to grow in the presence of these extracts albeit at a slower rate. This indicates that these isolates are capable of growing in the presence of these compounds and may be capable of growing through and improving the permeability of heartwood material.

4) Despite concerns over the effect of moisture levels and inhibition by the tree defence mechanisms *Trichoderma* isolates were readily established into freshly felled Scots pine and Sitka spruce material that had moisture contents of around 120 and 220% respectively. Consequently it can be concluded that pole material can be inoculated with such permeability enhancing agents shortly after felling.

5) As expected air permeability of Scots pine was significantly higher than Sitka spruce. After exposure of Scots pine and Sitka spruce sapwood and heartwood cores to selected *Trichoderma* isolates however the permeability of these samples was significantly increased. Unfortunately the values of permeability achieved after treatment of the spruce cores did not however reach those of untreated pine cores and it can be concluded that despite increases of up to 3-5000% the permeability of the Sitka spruce remains too low to allow adequate treatment for poles. This means that although the treatment has improved the permeability by pit degradation and removing material from the rays Sitka spruce still has too few effective routes of ingress to allow pressure impregnation of preservative. It would be worthy of further investigation to determine if a different form of treatment e.g. oscillating pressure would have resulted in better treatment in the *Trichoderma* treated material.

6) The organisms ability to improve the permeability of the timber varied dependent on the different delivery systems used for inoculating the logs with *Trichoderma*. Timber inoculated using spore suspensions resulted in better overall increases in permeability and pellet inoculum gave poorer increases in permeability particularly at the ends of the logs furthest from the inoculation site. Ideally a delivery system should promote rapid growth and colonisation of the material over as large an area as possible. This may be improved by the addition of nutrients to enhance growth or compounds which would favour the colonising organism and give it an advantage over competing organisms. In this series of experiments a spore suspension was either sprayed or painted onto the surface of the wood material, further improvements may be to use a dipping process or by the application of a paste to the outside of the material.

7) Covering pole sections during *Trichoderma* treatment led to greater creosote loadings after treatment although this may be due to problems of drying in uncovered pole material.

8) Although drying patterns differed significantly between spruce and pine during seasoning, pole sections treated with *Trichoderma* showed no measurable differences in drying time when compared to untreated control material.

9) Creosote penetration into spruce poles inoculated with *Trichoderma aureoviride* SIWT1 was significantly higher than control or pole sections treated with *T. viride* SIWT 70 however the depth of penetration remains below that required to produce a pole product capable of providing a reasonable service life. Penetration into pine pole sections showed no significant difference between the treatments as full penetration of the sapwood was observed in all cases.

10) Poles inoculated with *Trichoderma* isolates showed the highest loadings of preservative in the treated area, for both Scots pine and Sitka spruce. Loadings in pine treated with *T. viride* SIWT 70 treated sections were up to 3 times those recorded in

similar control material. The significance of this is that the extra creosote may give a better treated product, but excessive creosote may have environmental repercussions with poles bleeding and preservative entering the environment around the pole. The increase in permeability of the timber may ultimately require an adjustment to the pressure treatment process as lower pressures and cycle times may be required to treat the timber effectively. Reductions in processing times and energy consumption may result in savings in the production and treatment costs of the timber. Financially the greatest savings could be made in reducing the seasoning time of the timber.

11) Radial profiles of preservative in the pine pole sections showed highest concentrations of preservative in the outer layers of the sapwood. Of all the treatments, sections treated with *Trichoderma viride* SIWT 70 showed the highest creosote retentions in these outer regions which should maximise resistance against soft rot. The outer regions of the treated timber are those that are most likely to come in contact with decay organisms, the higher concentrations of the preservative would prevent colonisation of the organisms in the timber and act as a protective barrier for the timber.

12) It is clear that natural improvements in wood permeability do occur during normal seasoning, since material treated with anti-sapstain chemicals to eliminate fungal colonisation consistently gave lower penetrations and preservative retentions when compared with other non-*Trichoderma* treated control material.

13) On the basis of careful selection *Trichoderma* isolates if delivered properly can be used to improve the permeability of both pine and spruce leading to enhanced treatment for use as pole material.

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Appendix 1

Pectinase Activity for Selected *Trichoderma* Isolates

Isolate	P-G			P+G	
Trichoderma aureoviride SIWT.1	73	70.4		16.35	17.05
T. harzianum IMI 206040	67.2	71.2		0	0
T. polysporum IMI 206039	81	87		14.5	14.1
T. viride IMI 24039	57.23	49.43		15.2	16.4
T. viride IMI 49791	67.2	69.46		6.9	6.5
Trichoderma CCA sample	45.9	54.1		5.8	5.4
Trichoderma FYT strain	53	59.5		0	0
T. viride IMI 335517	22	18		14.6	13.8
T. harzianum IMI 335518	68.9	71.1		18.3	16.9
T. pseudokoningii SIWT. 22	55.69	58.31		0	0
T. harzianum SIWT. 25	57.6	62.4		12.8	13.8
T. pseudokoningii SIWT. 33	62.3	61.1		7.1	5.4
T. pseudokoningii SIWT. 51	33.3	33.3		0	0
T. pseudokoningii SIWT. 55	46	46.8		0	0
T. pseudokoningii SIWT. 64	67.3	62.7		22.1	24.9
Trichoderma SIWT. 140	49.3	50.7		7.1	5.4
Trichoderma (ASH)	48.3	45.9		5.9	5.9
T. citrinoviride IMI 335519	31.8	33.2		0	0
T. saturnisporum IMI14685	36.2	33.8		12.7	12.3
Trichoderma viride SIWT. 11	66.5	63.5		15.3	16.3
T. viride SIWT. 14	8	8.4		0	0
T. viride SIWT. 24	44.8	42.6		11.3	10.9
T. viride SIWT. 30	27.5	28.5		6.3	7.1
Trichoderma SIWT. 38	18.3	19.1		0	0
T. viride SIWT. 43	24.7	21.9		6.9	6.5
T. viride SIWT. 53	17.3	18.7		11.7	10.5
T. viride SIWT. 70	82.5	90.9		13.6	11.4
T. viride SIWT. 90	37.6	40.2		6.75	6.65
Trichoderma SIWT. 170	22.6	23.4		13.2	13.4
T. polysporum SIWT. 220	21.3	22.7		6.3	6.2
T. hamatum SIWT. 4	32.3	33.7		0	0
T. polysporum SIWT. 13	23.6	24.4		0	0
T. saturnisporum SIWT. 91	22.8	19.2		6.5	7.7
T. hamatum SIWT. 44	24.1	25.9		24.3	25.7
T. saturnisporum SIWT. 69	51.8	48.2		6.8	6.6

The above shows the % loss in viscosity of a 2% solution of pectin after 2hrs incubation with the filtrate containing extracellular enzymes from selected *Trichoderma* isolates. P-G represents results where growth media did not contain added glucose. P+G represents results where growth media contained added glucose.

Appendix 2

Amylase Activity for Selected *Trichoderma* Isolates

Isolate	A+G			A-G	
Trichoderma aureoviride SIWT.1	0	0		0.43	0.57
T. harzianum IMI 206040	0	0		0.44	0.52
T. polysporum IMI 206039	0	0		1.2	1.56
Trichoderma FYT strain	0.16	0.2		0.95	1.25
T. harzianum IMI 335518	0	0		0.11	0.03
T. pseudokoningii SIWT. 22	0	0		1.23	1.77
T. harzianum SIWT. 25	0.58	0.76		0.49	0.53
T. pseudokoningii SIWT. 33	0	0		0.31	0.43
T. pseudokoningii SIWT. 51	0	0		1.5	1.42
T. pseudokoningii SIWT. 55	0	0		0.51	0.67
T. pseudokoningii SIWT. 64	0	0		0.83	0.87
Trichoderma SIWT. 140	0	0		0.91	0.75
Trichoderma (ASH)	0	0		1.36	1.14
T. citrinoviride IMI 335519	0	0		0.41	0.49
T. saturnisporum IMI14685	0	0		0.28	0.38
T. longibrachiatum IMI 536408	0.27	0.25		0.09	0.11
T. reesei IMI 192656ii	0.55	0.61		1.15	1.45
T. viride SIWT. 53	0	0		0.46	0.64
T. viride SIWT. 60	0	0		0.45	0.33
T. viride SIWT. 70	0	0		0.89	1.09
T. viride SIWT. 90	0	0		1.21	1.03
T. viride SIWT. 100	0	0		0.43	0.73
Trichoderma SIWT. 170	0.3	0.2		3.84	3.68
T. polysporum SIWT. 220	0.1	0.16		0.2	0.2
T. saturnisporum SIWT. 91	0	0		1	0.98

The above shows the concentration (mg/ml) of reducing sugar released from a starch after incubation with the selected *Trichoderma* isolates. A-G represents results where growth media did not contain added glucose. A+G represents results where growth media contained added glucose.

Appendix 3

Cellulase Activity for Selected *Trichoderma* Isolates

Isolate	c+g			c-g	
Trichoderma aureoviride SIWT.1	60.1	68.7		44.1	44.1
T. harzianum IMI 206040	41	39		38.6	37.8
T. polysporum IMI 206039	41.5	38.5		30.2	28.6
T. viride IMI 24039	33.9	37.3		41.8	40.6
T. viride IMI 49791	54.2	57		46.2	42
Trichoderma CCA sample	46.1	42.7		26.9	26.1
Trichoderma FYT strain	63	61.4		29.8	29
T. viride IMI 335517	31.8	30.4		43	39.4
T. harzianum IMI 335518	61	59		36.1	34.5
T. pseudokoningii S.I.W.T 22	61.5	62.9		33.2	31.6
T. harzianum S.I.W.T 25	70.2	67.6		29.5	29.3
T. pseudokoningii S.I.W.T 33	27.6	30.2		32.7	32
T. pseudokoningii S.I.W.T 51	51.1	55.5		41	41.4
T. pseudokoningii S.I.W.T 55	66.6	71.2		43	39.4
T. pseudokoningii S.I.W.T 64	61.2	63.2		26.4	26.6
Trichoderma S.I.W.T 140	41.3	43.1		38.6	37.8
Trichoderma (ASH)	62.3	66.5		40.9	41.5
T. citrinoviride IMI 335519	64.8	59.6		28.9	29.9
T. saturnisporum IMI14685	59.3	56.3		40.6	41.8
T. longibrachiatum IMI 536408	50.6	51.6		16.4	15.2
T. reesei IMI 192656ii	44.1	44.7		26.6	26.4
T. saturnisprorum S.I.W.T 142	40	40		20.8	20.4
Trichoderma viride SIWT11	44.3	44.5		43.6	44.6
T. viride S.I.W.T 14	41.2	43.2		34	30.8
T. viride S.I.W.T 24	43.2	44.2		38.3	38.1
T. viride S.I.W.T 28	41	39		38.4	38
T. viride S.I.W.T 30	56.3	59.3		31.3	33.5
Trichoderma S.I.W.T 38	39.2	40.8		30	28.8
T. viride S.I.W.T 40	44.6	44.2		46.3	47.7
T. viride S.I.W.T 43	42.3	42.1		35.2	35.4
T. viride S.I.W.T 53	55.8	55.2		33.5	31.1
T. viride S.I.W.T 60	38.2	37.4		43.6	44.6
T. viride S.I.W.T 67	43.7	45.1		40.1	36.3
T. viride S.I.W.T 70	67.5	65.9		41.9	40.5
T. viride S.I.W.T 90	73.3	68.9		36.4	34
T. viride S.I.W.T 100	61.5	67.3		38.2	38.2
T. viride S.I.W.T 110	50	47.6		42.9	45.3
T. hamatum S.I.W.T 150	63.5	65.3		38.5	37.9
Trichoderma S.I.W.T 170	52	50.2		34.7	35.9
Trichoderma S.I.W.T 190	61	63.4		39.3	37.1
T. polysporum S.I.W.T 220	41.9	42.5		38.5	37.9
T. hamatum S.I.W.T 4	75	71.6		46.7	47.3
T. polysporum S.I.W.T 13	49.3	48.5		32.9	31.8
T. saturnisporum S.I.W.T 91	38.2	37.4		47.6	46.6
T. hamatum S.I.W.T 44	26.5	26.9		38.7	37.7
T. polysporum S.I.W.T 200	48.3	49.5		46.3	47.9
T. saturnisporum S.I.W.T 69	41.5	42.9		46.2	42

The above shows the % loss in viscosity of a 2% solution of Cellulose after incubation with the selected *Trichoderma* isolates. C-G represents results where growth media did not contain added glucose. C+G represents results where growth media contained added glucose.

Appendix 4

For a matched pair (a & b) of wood blocks a) Treated with *Trichoderma* b) Untreated control. Both have dry weights of 5g.

After dipping in decalin for 10 secs a = 10 g and b = 8g

therefore:

1) Gross increase in decalin uptake:

Weight (g) decalin taken up by *Trichoderma* treated block - Weight (g) decalin taken up by matched control.

ie 10g -8g = 2g

2) Mean increase in % uptake of decalin:

Calculated as follows:

% increase in decalin uptake in *Trichoderma* treated block* - % increase in decalin uptake in corresponding control block*.

ie (10-5)/5 x100 = 100% (8-5)/5 x100 = 60%

therefore increase: 100%-60% = 40% data is then pooled for replicate samples.

3) Mean percentage increase in uptake (%) of decalin. Calculated as follows result of determination 2) divided by % uptake for control block.

ie from above % increase = 40% / 60% from control block = 66 % increase

again data is then pooled for replicate samples.

Appendix 5

Dehydrogenase Activity and deviations for Scots pine and Sitka spruce sapwood from pole sections treated with selected *Trichoderma* isolates.

Wood type	Treatment	1 Week		6 Weeks		10 Weeks	
		Ave	St Dev	Ave	St Dev	Ave	St Dev
	Uncovered T35	0.8	0.19	1.54	0.41	0.49	0.32
	Covered T35	1.7	0.6	1.42	0.11	1	0.47
Scots Pine	Uncovered T1	0.7	0.29	1.54	0.27	1.41	0.17
	Uncovered T1	1.36	0.26	1.44	0.42	0.53	0.34
	Uncovered T35	0.98	0.155	1.02	0.09	0.27	0.16
	Covered T35	1.13	0.31	1.16	1.56	0.87	0.67
Sitka spruce	Uncovered T1	1.13	0.63	1.31	0.23	0.27	0.2
	Uncovered T1	1.39	0.23	0.87	0.15	0.533	0.123
		16 Weeks		24 Weeks		35 Weeks	
		Ave	St Dev	Ave	St Dev	Ave	St Dev
	Uncovered T35	1.11	0.26	2.08	1	0.7	0.06
	Covered T35	1.93	1.11	2.17	0.27	0.9	0.33
Scots Pine	Uncovered T1	0.81	0.19	1.29	0.16	0.69	0.16
	Uncovered T1	1.31	0.16	1.95	0.51	1.12	0.27
	Uncovered T35	0.19	0.17	0.43	0.4	0.26	0.25
	Covered T35	1.14	0.23	1.08	0.43	0.6	0.37
Sitka spruce	Uncovered T1	0.53	0.17	0.8	0.23	0.43	0.32
	Uncovered T1	0.77	0.29	0.99	0.62	0.7	0.14

Appendix 6

Calculation of corrected preservative loadings for pole sections.

Example: Covered Sitka spruce pole sections treated with *Trichoderma aureoviride.*

Average length= 2meters

Average volume = 0.07 m^3

Average penetration = 0.015 m

Average retention of creosote in the treated area = 95 kg/m^3

Average radius = 0.1055 m.

From the above the average volume of the untreated pole section = 0.05 m^3

Hence the volume of the treated region = 0.02 m^3

Therefore 95kg/ m^3 is in 0.02 m^3 giving a total weight of 1.9kg for the pole section.

Therefore the corrected loading for the whole pole section is 27kg/ m^3 .